THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

VI. The Blood Picture at Various Periods after Splenectomy.*

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In an earlier communication one of us[1] has described the changes in the blood observed in three dogs during a period of three months after splenectomy. The observations indicate that a secondary anemia develops almost immediately, reaches its greatest severity at the end of about one month, and that the blood picture returns to normal after about two and a half to three months. Accompanying this change in the red cells is a leucocytosis most marked immediately after operation, and diminishing gradually, though persistent to a slight degree, as long as counts were made. The only other point of interest was the absence of eosinophils from the third to the eleventh day, but followed later by an increase varying from 6 to 20 per cent. of the total white cell count.

Later, in a second investigation,[2] the object of which was to determine the relation between various grades of anemia and the ease of producing hemolytic jaundice, it was found that the blood picture after splenectomy is not always the same. Sometimes the anemia develops very slowly, or is of only mild degree, or is long continued with the reparative process coming on very late. These

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[1] Musser, J. H., Jr., An Experimental Study of the Changes in the Blood Following Splenectomy, *Arch. Int. Med.*, 1912, ix, 592.

[2] Pearce, R. M., Austin, J. H., and Musser, J. H., Jr., The Relation of the Spleen to Blood Destruction and Regeneration and to Hemolytic Jaundice. III The Changes in the Blood Following Splenectomy and Their Relation to the Production of Hemolytic Jaundice, *Jour. Exper. Med.*, 1912, xvi, 758.

results appeared to demand a supplementary study on a larger number of animals and for longer periods. It is this study that is here presented.

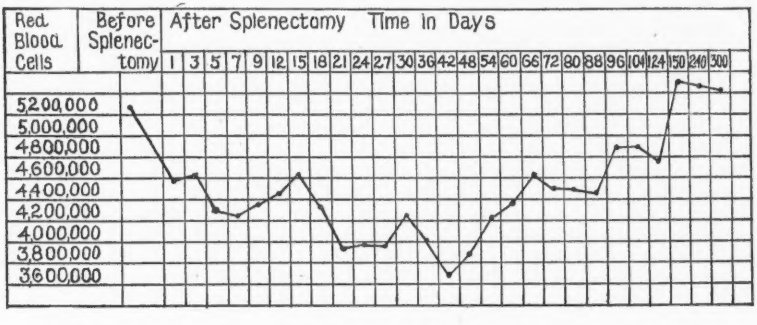
MATERIAL.

In addition to continuing the counts forming the basis of the first report, counts have been made also on four other animals; thus this report is based on observations on seven animals, six of which were under observation for periods of eight to ten months after splenectomy.

The operation in each case was a simple, practically bloodless, splenectomy under ether anesthesia. The animals were kept under as favorable circumstances as kennels allow, and as a rule showed an increase in weight at the end of ten months, though two (Nos. 32 and 33) dropped, respectively, from 10,260 and 7,050 grams to 9,170 and 5,980 grams.

RESULTS.

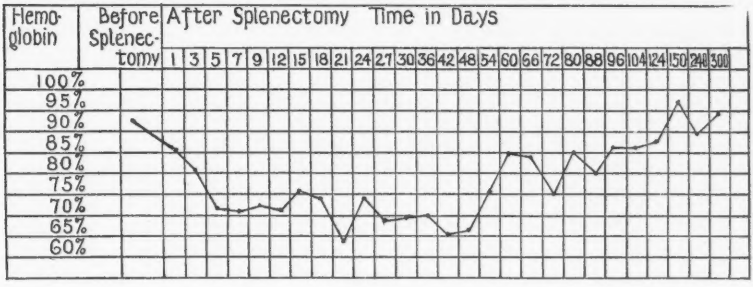
The changes in the red cell count, the hemoglobin content, and the leucocyte count, respectively, are shown in tables I, II, and III, while in text-figures 1, 2, and 3 are shown the corresponding composite curves.



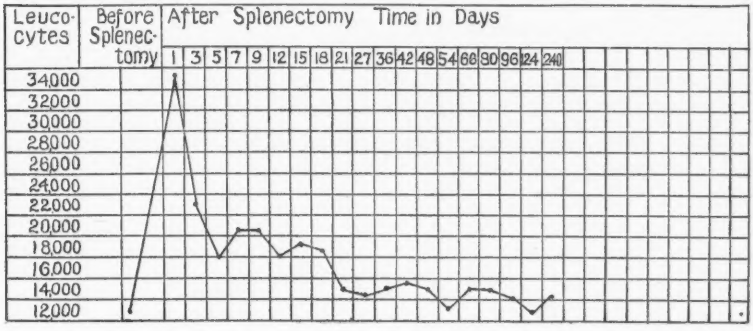
TEXT-FIG. 1. Composite curve of the red blood cell count of seven dogs.

As will be readily seen, this larger number of observations for longer periods shows greater variations than was evident in the first series. In general the anemia comes on promptly, reaches its

maximum severity after about thirty to forty-five days, and then after gradual improvement the red cell count approaches the normal level at about the end of three or four months, but continues to increase until the end of the eighth or tenth month. An



TEXT-FIG. 2. Composite curve of the hemoglobin estimation of seven dogs.



TEXT-FIG. 3. Composite curve of the leucocyte counts of five dogs.

exception is seen in dog 44, in which the red cell count and hemoglobin content had not returned entirely to normal after ten months, though the initial anemia was well marked at the fifteenth day. On the other hand, dog 46 failed to show a fall in the red cell count until one month had elapsed.

As a rule, the decrease in hemoglobin content occurs a little sooner and is more marked than the fall in the red cell count; also as improvement begins the increase in hemoglobin falls behind the increase in red cells.

The hemoglobin figures vary considerably, the lowest being 50 to 60 per cent. during the fourth to the seventh week, the drop before this time and the rise after being equally gradual.

TABLE I.

Red Cell Count.

Preliminary count, days.	Dog 32.	Dog 33.	Dog 41.	Dog 44.	Dog 46.	Dog 57.	Dog 59.
	5,340,000	4,930,000	5,350,000	5,150,000	5,520,000	5,520,000	4,610,000
1	4,500,000		4,540,000	4,450,000		5,100,000	4,140,000
3	4,700,000		4,710,000	5,090,000		5,120,000	3,690,000
5	4,470,000		4,700,000	4,860,000		5,125,000	2,390,000
7	4,620,000		4,070,000	5,120,000			3,120,000
9	4,800,000		3,920,000	5,060,000		3,940,000	3,960,000
12	4,610,000		3,840,000	4,310,000	5,820,000	3,810,000	4,210,000
15	4,600,000	5,980,000	3,500,000	3,720,000	5,590,000	4,280,000	
18	3,900,000		3,200,000	3,500,000	6,310,000	4,440,000	4,600,000
21				3,390,000		4,390,000	
24	3,550,000		2,890,000		5,760,000	3,150,000	4,460,000
27				3,220,000	5,380,000		3,560,000
30		4,790,000	3,050,000		5,280,000	3,560,000	4,120,000
36	3,720,000			3,100,000	4,960,000	2,970,000	4,510,000
42		3,550,000	2,880,000		4,580,000	3,200,000	4,010,000
48	3,970,000	4,600,000	3,010,000	3,680,000		4,100,000	4,490,000
54	4,240,000	5,020,000	3,260,000			4,210,000	4,570,000
60		4,000,000				4,740,000	
66	4,890,000	4,190,000	3,980,000	4,740,000		5,010,000	4,980,000
72		4,300,000				4,680,000	4,700,000
80	5,350,000	3,680,000	4,950,000	4,740,000		5,980,000	
88		3,860,000				5,320,000	4,340,000
96		4,000,000				5,480,000	5,100,000
104	5,100,000	4,200,000	5,240,000	4,500,000			5,400,000
124	5,230,000	4,340,000	4,610,000	4,150,000		5,240,000	
150		4,800,000				5,216,000	6,250,000
240	6,120,000	5,184,000	5,230,000	4,460,000		5,100,000	5,550,000
300		5,350,000	5,490,000	4,970,000		6,050,000	

The leucocyte picture is quite constant in all animals. All developed a leucocytosis of 26,000 to 38,000 per cubic millimeter of blood on the day after splenectomy, with a rapid fall in a few days to 20,000, and then a more gradual decrease, reaching almost to the normal level at the end of four months.

It was found that the initial leucocytosis was due mainly to an increase in polymorphonuclear neutrophilic leucocytes. An eosinophilia of from 10 to 32 per cent. persisted in one dog during 113 days of observation, while in another it varied during 107 days from 6 to 11 per cent. Lymphocytosis, as has been usually de-

scribed, was not found in this series. In no count were the lymphocytes recorded as higher than 32 per cent., while the average count in all dogs was about 18 to 26 per cent. As, therefore, computations of the actual number of the different white cells per cubic millimeter would show much the same results as the percentages given, the figures are not presented. Punctated normocytes, such as are described by Hörz,[3] were not found.

TABLE II.

Hemoglobin Estimation.

Preliminary estimation, days.	Dog 32.	Dog 33.	Dog 41.	Dog 44.	Dog 46.	Dog 57.	Dog 59.
	100 per cent.	98 per cent.	90 per cent.	105 per cent.	88 per cent.	94 per cent.	80 per cent.
1	90		70	92		105	70
3	85		70	92		95	64
5	80		70	82		83	48
7	80		70	82			55
9	80		70	72		77	65
12	75		70	72	85	62	65
15	70	88	70	62	98	70	
18	65		70	60	104	77	70
21	65		60	58		70	
24	65				98	71	66
27			50	55	96		63
30	70	80	55	57	95	55	74
36					68	50	70
42	70	52	55	70	75	61	80
48		58	55			76	76
54	80	78	65	72		84	74
60		82				89	
66	90	78				90	78
72		70	70	75		84	75
80	90	79	90			82	
88		78		80		94	72
96	90	66	85	90		97	88
104		62					94
124	95	75	80	95		90	
150		94	85			110	105
240		100	75	68		104	105
300	97	102	92	81		108	

In our first communication[4] on this subject, the conflicting reports of the literature were given. The few reports that have appeared since then support the general findings; that is, that after splenectomy there is a distinct anemia with hyperleucocytosis and transient

[3] Hörz, W., Ueber Splenektomie bei traumatischer Milzruptur, *Beitr. z. klin. Chir.*, 1906, l, 188.

[4] Musser, J. H., Jr., *loc. cit.*

eosinophilia. Schulze,[5] for example, by daily counts for forty-seven days, followed the blood changes in man that occurred after a posttraumatic splenectomy, and found that the red cells after dropping quickly to three million per cubic millimeter remained below four million for a month and a half. After four months the erythrocyte count had risen to 5,200,000. The hemoglobin, after

TABLE III.

Leucocyte Count.

Preliminary count, days.	Dog 32.	Dog 41.	Dog 44.	Dog 57.	Dog 59.
	9,000	12,000	13,000	14,400	14,200
1	26,000	33,000	36,000	38,100	28,700
3	26,000	22,500	21,500	21,100	13,800
5	28,000	21,000	13,000	17,000	11,400
7	25,500	23,000	13,000		17,900
9	28,000	19,000	14,000	22,000	14,400
12	22,000	21,000	13,000	19,200	17,800
15	25,000	18,000	16,000	19,600	
18	19,000	19,000	18,100	18,700	16,800
21	18,000	14,000	16,400	18,400	
24	15,000			18,600	12,100
27		15,000	12,000		11,800
30	33,000	15,000	14,200	16,000	13,400
36				16,900	15,300
42	16,000	11,000	11,200	19,000	12,100
48		12,000		19,200	17,400
54	11,000	11,000	10,000	15,000	13,100
60				16,100	
66	13,000			17,600	11,600
72		12,000	11,200	16,400	12,200
80	14,000	12,000		16,000	
88			9,900	18,100	13,600
96	13,000	13,000	11,000	20,100	9,000
104					
124	15,000	13,000	12,400	16,000	
240				16,400	15,000

staying at 90 per cent. for a few days, dropped to 70 and was at 80 after forty-seven days; after four months, however, it was only 81 per cent. The leucocytes rose quickly to 20,000 (on two counts there were almost 30,000); after forty-seven days there were 10,000, and after four months 9,300. An eosinophilia of 5 to 8 per cent. persisted for seventeen days and then dropped to normal.

[5] Schulze, F., Beitrag zur Splenektomie bei der traumatischen Milzruptur und zur Frage der dadurch bedingten Blutveränderungen, *Beitr. z. klin. Chir.*, 1911, lxxiv, 456.

In attempting to explain the gradual return toward normal after splenectomy it is assumed by the investigators who consider the spleen a hemopoietic organ, that other tissues, such as the lymph or hemolymph nodes, take on the activities of the spleen. In this connection the finding by Faltin[6] of multiple spleen-like bodies, having histologically the structure of the spleen, would appear to offer some support to this explanation. These supernumerary spleens are not, however, always found in man, when post-mortem examinations have been made long after splenectomy, and we have found no evidence of such compensatory development in the dog one year after splenectomy. We have no explanation to offer either of the anemia that follows splenectomy or of the later blood regeneration. These two problems, as well as that concerning the increased resistance of the red blood corpuscles, always associated with the anemia of splenectomy, have been and still are under investigation in this laboratory,[7] but with little prospect of an immediate solution.

SUMMARY.

After splenectomy anemia usually develops quickly and reaches its height in from three to six weeks; then with gradual improvement the blood picture approaches the normal after about three to four months, with complete return to normal in five to ten months. Accompanying this is marked leucocytosis reaching its height in twenty-four hours but persisting to a slight degree for several months. Variations, however, may occur, in that the anemia may develop slowly and be of slight degree, or the reparative process may be delayed and up to ten months incomplete. The anemia is, however, inevitable, as is also, later, some degree of repair.

[6] Faltin, R., Milzartige Bildungen im Peritoneum, *Deutsch. Ztschr. f. Chir.*, 1911, cx, 160.

[7] Pearce, R. M., Austin, J. H., and Krumbhaar, E. B.; Pearce, R. M., Austin, J. H., and Musser, J. H., Jr.; Pearce, R. M., Austin, J. H., and Musser, J. H., Jr.; Karsner, H. T., and Pearce, R. M.; Pearce, R. M., and Austin, J. H.; The Relation of the Spleen to Blood Destruction and Regeneration and to Hemolytic Jaundice, I to V, *Jour. Exper. Med.*, 1912, xvi, 363, 375, 758, 769, 780.

THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

VII. The Effect of Hemolytic Serum in Splenectomized Dogs.*

By RICHARD M. PEARCE, M.D., and MAX MINOR PEET, M.D.

(*From the John Herr Musser Department of Research Medicine of the University of Pennsylvania, Philadelphia.*)

In our previous investigations[1] of this general subject, the lessened tendency to hemolytic jaundice and the increased resistance of the red cells after splenectomy have been described. In connection with both of these phenomena it is essential to know whether they persist for long periods after splenectomy, and it is also desirable that some explanation of the mechanism of each be found. In the present communication we present additional data on these two problems.

THE REACTION TO HEMOLYTIC SERUM IN DOGS SPLENECTOMIZED ONE YEAR.

With few exceptions all experiments previously reported have been on dogs splenectomized for relatively short periods, and in the three instances in which the period was a long one (7½, 9, and 10 months) the animals quickly succumbed after 8, 18, and 24 hours, respectively, to the general toxic effect of the serum. That this is not a constant result is shown in the following experiments (table I), which add also to the evidence concerning the increased resistance of the red cells and the decreased tendency to hemolytic jaundice after splenectomy.

In this experiment the effect of the hemolytic serum is very much the same in the splenectomized as in the normal dog, as shown by

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[1] *Jour. Exper. Med.*, 1912, xvi, 363, 375, 758, 769, 780; 1913, xviii, 487.

the blood count, but with a slightly more definite elimination of bile pigment in the normal dog. The difference is, however, so slight that on the basis of this experiment alone one might assume

TABLE I.

Effect of Hemolytic Serum One Year after Splenectomy, with Control.[2]

Date.	One year after splenectomy (dog 24).	Date.	Control (dog 22).
Feb. 10, 1912	Weight, 7,860 gm. Splenectomy.		
Feb. 20, 1913	Weight, 6,820 gm. Blood: red cells, 6,048,000; hemoglobin, 110 per cent. Fragility: 0.25+,[3] 0.4−. Urine: normal.	Feb. 20, 1913	Normal dog; weight, 7,740 gm. Blood: red cells, 6,470,000; hemoglobin, 105 per cent. Fragility: 0.35+, 0.45−. Urine: normal.
Feb. 21	Received in vein 0.285 c.c. hemolytic serum (No. 64) per kilo.	Feb. 21	Received in vein 0.25 c.c. of same serum per kilo.
Feb. 22	Urine: normal.	Feb. 22	Urine: contains bile.
Feb. 23	Urine: faint trace of bile pigment.	Feb. 23	Urine: contains bile; well marked test.
Feb. 24	Blood: red cells, 5,664,000. Urine: normal.	Feb. 24	Blood: red cells, 5,760,000. Urine: normal.
Feb. 24, 12 M.	Received 2d injection of same serum, 1 c.c. per kilo.	12 M.	Received 2d injection of same serum, 1 c.c. per kilo.
3 P.M.	Vomited. Urine by catheter contains bile; trace only.	3 P. M.	Vomited. Urine contains bile; trace only.
Feb. 25	Blood: red cells, 3,568,000. Urine: small amount of hemoglobin present; also faint trace of bile.	Feb. 25	Blood: red cells, 3,200,000. Urine: large amount of hemoglobin; bile well marked.
Feb. 26	Urine: no hemoglobin; trace of bile.	Feb. 26	Urine: trace of bile.
Feb. 27–28	Urine: faint trace of bile.	Feb. 27–28	Urine: faint trace of bile.
March 1, 2, 3	Urine: faint trace of bile.	March 1–2 March 3	Urine: no bile. Urine: trace of bile.
March 4	Urine: no bile. Chloroformed.	March 4	Urine: no bile. Chloroformed.

that after a year of splenectomy the peculiarities due to splenectomy had disappeared and that the animals react in the same way as the normal dog. That this is not the usual occurrence is shown in table II.

[2] The same serum, No. 64, was used in both animals.

[3] + indicates complete hemolysis; —, no hemolysis.

TABLE II.

Effect of Hemolytic Serum One Year after Splenectomy, with Control.[4]

Time.	One year after splenectomy (dog 42).	Time.	Control (dog 43).
Before injection.	Weight, 6,400 gm. Urine: no albumin, no bile.	Before injection.	Weight, 6,615 gm. Urine: no albumin, no bile.
1st injection.	Received in vein 0.5 c.c. serum per kilo.	1st injection.	Received same dose of same serum as dog 42.
After injection.	Urine: trace of albumin;	After injection.	Urine: trace of albumin;
1–3 dys.	faint trace of bile.	1 dy.	well marked bile test.
		2–3 dys.	Urine: no bile.
3 dys.	Received in vein 1 c.c. of	3 dys.	Received same dose of
2d injection.	same serum per kilo.	2d injection.	same serum as dog 42.
4 dys.	Marked hemoglobinuria.	4 dys.	Marked hemoglobinuria.
5 dys.	Faint hemoglobinuria; doubtful bile test.	5 dys.	Moderate hemoglobinuria; marked bile test.
6 dys.	Faint hemoglobinuria; faint but definite bile test.	6 dys.	Faint hemoglobinuria; large amount of bile.
	Died. No evidence of jaundice at autopsy.	7 dys.	Urine: no hemoglobin; deeply bile-stained. Chloroformed. At autopsy general bile staining of tissues.

This experiment shows the typical difference in the degree of hemolytic jaundice, in the splenectomized as compared with the normal animal, which has been observed in all our studies of this subject, and demonstrates conclusively, that lapses of time, up to one year at least, do not cause the disappearance of this phenomenon. As additional proof of the lessened tendency to hemolytic jaundice, despite the presence of hemoglobinemia, the following account of the administration of hemolytic serum eleven and a half months after splenectomy is presented, without notes as to the control.

TABLE III.

Time.	11½ months after splenectomy (dog 33).	Time.	11½ months after splenectomy (dog 33).
Before injection.	Weight, 5,975 gm. Blood: red cells, 5,350,000; hemoglobin, 102 per cent. Urine: no albumin, no bile.	2 dys.	Hemoglobinuria slight; faint trace of bile.
Injection.	Received in vein 0.5 c.c. hemolytic serum (No. 67) per kilo.	3 dys.	Blood: red cells, 3,800,000. Urine: no hemoglobin; faint but definite bile test; trace of albumin.
After injection. 1 dy.	Hemoglobinuria marked.	4–8 dys. 9–10 dys.	Urine: faint trace of bile. Urine: no bile. Experiment discontinued.

[4] The same serum, No. 65, was used in both animals.

SUMMARY.

The experiments demonstrate that dogs splenectomized for one year exhibit the same increased resistance of the erythrocytes and the same lessened tendency to hemolytic jaundice previously observed in animals splenectomized for shorter periods of time. One apparently discordant experiment (table I) suggests that possibly after a longer lapse of time than a year the lessened tendency to jaundice might not be so evident. On the other hand, it is in splenectomized animals having the high red cell and hemoglobin content of the animal representing this experiment, that, as we have shown elsewhere,[5] hemolytic jaundice may occur in equal degree to that of the control animal. We regard, therefore, the experiments here presented as in accord with those representing observations at earlier periods after splenectomy.

THE RETICULATED ERYTHROCYTES AND THEIR RELATION TO THE INCREASED RESISTANCE OF RED CELLS AFTER SPLENECTOMY.

With the introduction of vital staining many alterations in the structure of the red corpuscles have come to light. The most interesting of these, perhaps, are the "skeined" or reticulated corpuscles, which contain sometimes a few granules connected by fine threads, at other times a network of threads with many granules, or again a thick ball-like tangle of threads, demonstrable only by vital staining. They may be found in a very small number (0.5 per cent.) of the cells of normal adult blood, are more numerous in the blood at and shortly after birth, and are present in larger, but varying numbers in various pathological conditions.

As to their nature, it is generally accepted that they are probably analogous to the polychromatophilic cells of fixed preparations, but their relation to the common basic granulations of such preparations is still a matter of doubt. Concerning the question of their significance a voluminous literature is at hand; at first it was considered that the skeins represented a beginning degeneration, but the more recent opinion is almost unanimous that the skeins are to be found

[5] Pearce, R. M., Austin, J. H. and Musser, J. H., Jr., III. The Changes in the Blood Following Splenectomy and Their Relation to the Production of Hemolytic Jaundice, *Jour. Exper. Med.*, 1912, xvi, 758.

only in young erythrocytes and that an increased number of reticulated cells is evidence of unusual activity in blood production. It was therefore natural that the reticulated cells should be regarded as more resistant cells and that their presence should be interpreted as an explanation of the increased resistance of the red cells in various forms of anemia. The many and contradictory views on this subject may be found in the paper by Pepper and Peet,[6] who, working in this laboratory, investigated the question of the occurrence of reticulated cells in experimental anemia and the resistance of these cells to various hypotonic salt solutions. They reached the conclusion that although reticulation of the erythrocytes is evidence of the youth of the cell, no difference can be found between the normal and reticulated cells of a given animal in their resistance to hypotonic salt solution.

In the hope of showing some relation between these cells and the increased resistance of the blood of splenectomized animals, we have looked for these cells before and after splenectomy. It was assumed that an increase of the cells might explain the increased resistance of the red cells after splenectomy. The results, however, do not support this assumption. The examination[7] of several normal adult dogs showed that reticulated cells are absent or found only after prolonged search. On the other hand, they are no more numerous in dogs which have been splenectomized for 4, 20, and 54 days, and 3, 8, 10, and 12 months (2), than they are in normal dogs, and in two dogs which were followed closely for 44 and 56 days after splenectomy no changes in the number of reticulated cells could be determined.

The increased resistance of the red cells after splenectomy cannot be explained by an increase in the number of reticulated cells in the circulating blood.

[6] Pepper, O. H. P., and Peet, M. M., The Resistance of Reticulated Erythrocytes, *Arch. Int. Med.*, 1913, xii, 81.

[7] The technique used is as follows: A few drops of blood are caught directly from the point of puncture in a small test-tube containing 0.55 per cent. sodium chloride to which had been added two drops of a 2 per cent. solution of potassium oxalate and one drop of a saturated aqueous solution of brilliant cresyl blue. After standing a few minutes a drop from the bottom of the tube is removed with a pipette, placed on a slide and covered by a thin cover-slip. It is then examined with the oil immersion lens, and the number of skeined and unskeined cells are counted in a total of 500 cells.

CONCLUSIONS.

The increased resistance of the red blood corpuscles characteristic of the splenectomized animal is as evident one year after removal of the spleen as it is at earlier periods. So also is the decreased tendency to jaundice after the administration of hemolytic serum. The increase in resistance of the red cells cannot be explained on the basis of an increase in reticulated cells in the circulating blood.

A STUDY OF THE RELATION OF PULMONARY AND BRONCHIAL CIRCULATION.*

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A detailed report of the history of the lesser circulation is rendered unnecessary by the article of Tigerstedt.[1] Somewhat less well known is the history of the circulation in the bronchial vessels, but the earlier work has been carefully reviewed by Zuckerkandl[2] and also by Miller.[3] Much has been written, both descriptive and controversial, in reference to the physiology and anatomy of the two systems of vessels, and this literature is largely included in a recent paper by one of us.[4] In spite of the work of Miller[5] who claims that there is no real anatomical anastomosis other than by terminal capillaries between the two systems, the general opinion appears to be that functionally, at least, there is free exchange of bloods, a dictum of Virchow[6] practically contradicted in the earlier writings of Cohnheim and Litten,[7] who, however, retracted in later publications. In this period the work of Küttner[8] stands

* Aided by a grant from The Rockefeller Institute for Medical Research. Received for publication, June 2, 1913.

[1] Tigerstedt, R., Der kleine Kreislauf, *Ergebn. d. Physiol.*, 1903, ii, pt. 2, 528.

[2] Zuckerkandl, E., Ueber die Anastomosen der Vena pulmonalis mit den Bronchialvenen, etc., *Sitzungsber. d. k. Akad. d. Wissensch., math.-natur. Kl.*, 1881, lxxxiv, pt. 3, 310; Ueber Verbindungen zwischen den arteriellen Gefässen der menschlichen Lungen, *idem.*, 1883, lxxxvii, pt. 3, 171.

[3] Miller, W. S., Das Lungenläppchen, seine Blut- und Lymphgefässe, *Arch. f. Anat. u. Physiol., Anat. Abt.*, 1900, 197; The Arrangement of the Bronchial Blood Vessels (Preliminary Communication), *Anat. Anz.*, 1906, xxvii, 432; The Vascular Supply of the Pleura Pulmonalis, *Am. Jour. Anat.*, 1907, vii, 389.

[4] Karsner, H. T., and Ash, J. E., Studies in Infarction. II. Experimental Bland Infarction of the Lung, *Jour. Med. Research*, 1912, xxvii, 205.

[5] Miller, W. S., *loc. cit.*

[6] Virchow, R., Gesammelte Abhandlungen, Frankfurt, 1856, 295, experiment XX.

[7] Cohnheim, J., and Litten, M., Ueber die Folgen der Embolie der Lungenarterien, *Virchows Arch. f. path. Anat.*, 1875, lxv, 99. Litten, M., Untersuchungen über den haemorrhagischen Infarkt, und über die Einwirkung arterieller Anaemie auf das lebende Gewebe, *Ztschr. f. klin. Med.*, 1880, i, 131. Cohnheim, J., Vorlesungen über allgemeine Pathologie, Berlin, 1882, 207.

[8] Küttner, Beitrag zu den Kreislaufsverhältnissen der Froschlunge, *Virchows Arch. f. path. Anat.*, 1874, lxi, 21; Beitrag zur Kenntniss der Kreis-

out sharply as supporting the belief that there is extremely free interchange of blood and rich anastomosis between the two systems. More recently Königstein[9] has made a series of studies of the comparative anatomy and physiology of the two systems in the lungs of reptiles, birds, and amphibia, and has reached the same conclusions. Königstein lays much stress on the importance of the bronchial blood for the nutrition of the lungs. He states that it not only nourishes lung tissue in its own area of distribution, but also by mixing with the pulmonary blood can nourish more remote parts of the lung. This statement apparently is made on the basis of the fact that in certain of the animals which he examined the analogue of the bronchial artery can supply large amounts of blood for the nutrition of the lung, while the animal is not breathing. He further supports this statement by saying that under pathological conditions there appear no well marked alterations in the lung as a result of obstruction to branches of the pulmonary arteries because the bronchial arteries suffice for nourishment. This statement was made by Virchow in reference to obstruction of the main vessel to a lobe, but as has been shown by the work of Küttner and also by other work by one of us,[10] does not apply to obstruction in a smaller branch. Further studies are in progress which sustain our contention in this respect. The lack of serious disturbance to circulation when obstruction is present in a small branch of the pulmonary artery is due to the rich capillary and other anastomosis of the pulmonary itself rather than by anastomosis with the bronchial arteries. On the basis of injections of lungs Miller claims that coarse anastomosis between the bronchial and pulmonary arteries does not exist, although he admits that free interchange of fluids occurs when the injecting mass is of such a character that it will pass through capillaries.

In considering the function of the two systems the latter admission is sufficient, for if a capillary anastomosis is provided for free interchange of bloods, as Küttner conclusively pointed out, there is little need for discussion as to the exact size of the vessels that are concerned.

In order properly to appreciate the possibilities of the circulation in the lungs in a variety of pathological conditions, the following study was instituted, particularly with the idea of learning how great is the interchange between the two systems when the pressure in each is normal, and to what degree the mixture is changed under variations in the pressure in both systems. For this purpose, simultaneous injection of the two systems, under determined constant pressure, was considered necessary.

Mercury manometers and mercury safety valves were connected

laufsverhältnisse der Säugethierlunge, *Virchows Arch. f. path. Anat.*, 1878, lxxiii, 476.

[9] Königstein, H., Zur Morphologie und Physiologie des Gefäss-systems am Respirationstrakt, *Anat. Hefte*, 1903, xxii, 309.

[10] Karsner, H. T., and Ash, J. E., *loc. cit.*

with two compressed air outlets, and gelatin injection masses of carmine and of easily soluble Berlin blue were injected simultaneously, under measured pressures, into the pulmonary and systemic aortas of the dog. After orientation experiments it was found that 40 millimeters' pressure was the minimum pressure of good injection of the pulmonary artery and its capillaries, and 140 millimeters' pressure the minimum pressure of good injection of the bronchial arteries when injected by way of the aorta. In one series of dogs 40 millimeters was used as a constant pulmonary pressure, and pressures of 0, 40, 60, 100, 140, 160, 180, and 200 millimeters in the aorta. In a second series a constant pressure of 140 millimeters was used in the systemic aorta and pressures of 0, 10, 20, 30, 40, 60, and 80 millimeters in the pulmonary. Then with zero pressure in the aorta, pressures of 40 and 80 millimeters were used in the pulmonary, and with zero pressure in the pulmonary, pressures of 60, 140, and 180 millimeters were used in the aorta.

The technique was as follows: Under artificial respiration and complete ether anesthesia the dog's sternum was split[11] and the thorax held open with a self-retaining retractor. Ligatures were carefully placed about the pulmonary and aortic trunks near the heart, but not tied. Then the internal mammary arteries were ligated as a precautionary measure against subsequent leakage of the injection mass. To prevent free diffusion of the staining mass during injection, the brachiocephalic and left subclavian arteries (the two main branches of the aortic arch in the dog), the aorta just above the diaphragm and the inferior vena cava just below the heart were ligated. As quickly as possible cannulæ were inserted into the aorta and through the right ventricle into the pulmonary artery and tied in position by means of the previously placed ligatures. The cannulæ were filled to the tip with the injection mass before insertion and the pressures in each injection system regulated by means

[11] The dogs were normal except that immediately before opening the thorax five turnip radish seeds measuring from 2.5 to 3.5 mm. in diameter were placed in the right heart through a cannula in the jugular, so that the seeds lodged in smaller branches of the pulmonary artery. This was done so as to permit of an accessory study of the circulation in pulmonary embolism (Karsner, H. T., and Ghoreyeb, A. A., Studies in Infarction. III. The Circulation in Experimental Pulmonary Embolism, *Jour. Exper. Med.*, 1913, xviii, 507).

of the safety valves. A cannula was tied in the left ventricle and connected with an outlet tube. At a signal, the flow was started simultaneously in both systems, artificial respiration being continued and the flow maintained until the lungs ceased to collapse with the cut off pressure in the respiration apparatus. Then the outlet tube from the left ventricle was clamped and injection continued for a few minutes so as to make it complete. The tubing was clamped near the cannulæ and cut, the trachea clamped, the posterior part of the animal cut away, the head and thorax were placed in formalin (10 per cent.) in a refrigerator for forty-eight hours, and frozen sections of the lung were made for histological study. As a rule, considerable fluid appeared in the trachea before the end of the experiment, at first colorless, later considerably stained with the blue of the pulmonary injection mass.

The injection masses were made as follows: Twenty grams of gelatin were soaked in 300 cubic centimeters of cold water for thirty minutes. Twenty grams of easily soluble Grübler Berlin blue were dissolved in 1,200 cubic centimeters of water. The two were mixed in a hot water bath, after heating, and stirred until completely dissolved, then filtered and used on the same day. The carmine mass was made in the same proportions, but in order to dissolve the carmine it was necessary to add a small amount of strong ammonia water to the water of solution. The same process was followed and immediately before injection the solution was neutralized with acetic acid. Both masses were brought to 40° C. before injection.

Series 1.—Constant pressure of 140 mm. in the systemic aorta, and pressures of 0, 10, 20, 30, 40, 60, and 80 mm. in the pulmonary aorta. Of this series the fifth experiment combined the two minimal pressures of best injection. In this experiment the injection through the pulmonary was complete and perfect and the bronchial vessels also contained some of the blue injection mass. For the most part the bronchial vessels were filled with the red injection mass (aorta), and in the neighborhood of the larger bronchi (1 to 3 mm. calibre) the infundibular and alveolar capillaries were injected from the bronchial artery. With diminution of the pressure in the pulmonary artery down to as low as 10 mm., the injection of the pulmonary capillaries became somewhat less perfect, but remained fairly good. The injection of the bronchials was almost completely by the red mass (from the aorta) but still showed some injection from the pulmonaries. The tendency of the red mass to extend into pulmonary capillaries did not become more marked with diminu-

tion of the pulmonic pressure to 10 mm. With no pressure in the pulmonary artery it was possible after about fifteen minutes to inject from the aorta so that the pulmonary capillaries were almost completely injected.

Increase in pulmonary pressure to 60 mm. showed practically the same picture as that of 40 mm. pressure, and the same is true of the pulmonary pressure of 80 mm. The extension of the red to the neighboring alveoli is no less marked and the mass of blue (pulmonary injection mass) in the bronchial vessels is slightly greater than in the type experiment.

It would seem fair to state that the admixture of pulmonary and bronchial blood is very slight in lungs in which the pressure in the pulmonary is greater than zero and the pressure in the bronchials approximately normal. The slight extension of the bronchial circulation to the alveolar and infundibular capillaries is not influenced by variations in pulmonary pressure between ten and eighty millimeters. The mixture of bloods in the bronchial vessels varies slightly with the degree of pulmonary arterial pressure.

Series 2.—Constant pressure of 40 mm. in the pulmonary artery and pressures of 0, 40, 60, 100, 140, 160, 180, and 200 mm. in the aorta. The type experiment in this series is the same as in series 1, *i. e.*, pressures of 40 mm. in the pulmonary and 140 mm. in the aorta. With decreases in aortic pressure down to 40 mm. it was found that with aortic pressure of 100 mm. the picture remained practically the same as at 140 mm., and lower pressures showed no greater tendency of the red bronchial mass to pass into the alveolar or infundibular capillaries than with normal pressures; but, on the other hand, the blue pulmonary injection mass was found in increased amounts in the bronchial vessels. With no pressure in the aorta, the injection of the bronchial vessels by way of the pulmonary circulation was only partial. With increase of aortic pressure to 160, 180, and 200 mm., only after the pressure had reached 200 mm. was the extension of the bronchial injection mass into the infundibular and alveolar capillaries more marked. The mixture of the two masses in the bronchial vessels remained about the same.

It is apparent that when the bronchial pressure is lowered the pulmonary pressure when normal is sufficient partly to replace the bronchial circulation, but when the bronchial pressure is raised it does not tend to increase the circulation of bronchial blood in the pulmonary capillaries (alveolar and infundibular) until an extremely high pressure (200 millimeters) in the aorta is employed.

Series 3.—Constant pressure of zero in the pulmonary artery and pressures of 60, 140, and 180 in the aorta. With the lowest aortic pressure the bronchials showed complete injection, but there was practically no injection of the alveolar and infundibular capillaries except immediately in continuity with pulmonary vessels of a calibre of about 20 μ and larger. Pulmonary arteries

of 1 mm. and larger were not injected, although those smaller than 1 mm. in calibre were almost completely injected. A pressure of 140 mm. in the aorta, as has been stated, sufficed to inject, practically completely, the lung, and the same is true of a pressure of 180 mm. in the aorta.

Series 4.—Constant pressure of zero in the aorta and pressures of 40 and 80 mm. in the pulmonary artery. These animals showed complete injection of the pulmonary system. The lower pulmonary pressure resulted in partial injection of the bronchial vessels, whereas the higher pressure sufficed to inject almost completely the bronchial system, the submucous capillary loops being very well injected.

SUMMARY.

The most striking point brought out in this study is that as long as a definite pressure is maintained in either the pulmonary or bronchial circulations, the admixture of bloods is extremely limited. It is easily conceivable that more mixture occurs normally than under the conditions of the experiment, but there is no reason for considering this to be a large difference. If, however, in either system the pressure sinks to zero the possibility of supply by the other system becomes evident. It takes much longer for the mass injected through the bronchial arteries to penetrate to all parts of the lung than when the mass is injected through the pulmonary artery; but when accomplished, the injection reaches to all capillaries including those of the pleura, the only vessels remaining uninjected being the larger trunks of the pulmonary artery. On the other hand, the injection of the bronchial vessels by way of the pulmonary arteries is not complete with normal pressure, but occurs rapidly when a high pulmonary pressure is employed. It is therefore probable that either circulation can suffice for the simple nutritive demands of the lung if the other system is interfered with. It has been shown[12] that embolism of the pulmonary artery, without other circulatory disturbance, does not lead to necrosis of the affected area of the lung, but it is probable that the preservation of circulation is not due to collateral bronchial circulation so much as to the free anastomosis and early division into capillaries of the pulmonary artery. In support of this statement is the fact that the appearance is not altered when the bronchials are ligated at their origin. The same ligation shows no subsequent interference with the nutrition of the bronchi up to a period of five weeks, demonstrating

[12] Karsner, H. T., and Ash, J. E., *loc. cit.*

that the pulmonary circulation is sufficient to provide for the nutrition of the bronchi. If, however, as Virchow[13] has shown, the pulmonary artery supplying an entire lobe be occluded, the bronchial circulation can and does suffice for the nutrition of the lobe. In the case of the occlusion of a branch of the pulmonary artery the pressure in the area interfered with does not sink to zero because of the collateral circulation in this area; whereas, if the main trunk is occluded no collateral supply is available, the pressure sinks to zero, and the bronchial artery becomes available as a source of blood supply.

It must be remembered that the lung tissue, as a whole, has ready access to oxygen and this gas is the nutritive element acquired by the blood in the lungs. From these studies it would appear that the part of the lung tissue not in intimate contact with oxygen in the air is supplied by oxygenated blood of the bronchial arteries, and that the tissues through which the pulmonary blood circulates take up whatever organized nutriment they need from the pulmonary blood and possibly provide for their oxygen and carbon dioxide interchange (which must be very slight) either directly with the alveolar air, or by finding sufficient oxygen in the venous blood of the pulmonary artery.

The studies of the injected specimens confirm Küttner's findings of a very rapid breaking up of the pulmonary artery into capillaries.

In all the specimens studied it was found that although the pleural vessels can be injected by way of the bronchial arteries when there is zero pressure in the pulmonary arteries, yet when the two sets of vessels are injected simultaneously in the dog, the pleural vessels invariably derive their supply of injection mass from the pulmonary artery.

[13] Virchow, R., *loc. cit*

STUDIES IN INFARCTION.

III. The Circulation in Experimental Pulmonary Embolism.*

By HOWARD T. KARSNER, M.D., and ALBERT A. GHOREYEB, M.D.

(From the Laboratory of Pathology (Phillips Fund) of the Harvard Medical School, Boston.)

In an earlier paper[1] it was stated that "simple bland embolism of the pulmonary artery produces definite circulatory changes in the lung area supplied" (consisting chiefly of congestion) "provided this area extends along the sharp edge of the lobe and the embolus is of sufficient size, but no evidence of true infarction is to be found. So far as technical limitations permit, occlusion of the bronchial arteries makes no change in the circulatory alterations following simple embolism" of the pulmonary artery. This lack of participation of the bronchial artery was in apparent contradiction of an old experiment of Virchow's[2] in which he found that occlusion of the main trunk of the pulmonary was not followed by destruction of the lobe because the bronchial artery enlarged and supplied sufficient nourishment for the lobe. This experiment has been applied by many writers so as to explain the absence of true infarction following embolism of a smaller branch of the pulmonary artery; *i. e.*, by stating that the nutrition of the area supplied normally by the embolized pulmonary artery is taken over by the bronchial artery. In another paper[3] we have pointed out that in simultaneous injections of the two arterial systems there is almost no mixture of the bloods unless the pressure is zero in one or other of the two systems, and that when embolism of a smaller branch of the pulmonary artery occurs the pressure in the area supplied does not sink to zero because, as was shown in the first paper,[4] a circulation, independent of the bronchial circulation, is maintained, even though such circulation is considerably less than normal.

In order more thoroughly to understand what part the two circulations play in the case of embolism of the pulmonary artery the

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[1] Karsner, H. T., and Ash, J. E., Studies in Infarction. II. Experimental Bland Infarction of the Lung, *Jour. Med. Research*, 1912, xxvii, 205.

[2] Virchow, R., Gesammelte Abhandlungen zur wissenschaftlichen Medicin, Frankfurt a. M., 1856, 295, experiment XX.

[3] Ghoreyeb, A. A., and Karsner, H. T., A Study of the Relation of Pulmonary and Bronchial Circulation, *Jour. Exper. Med.*, 1913, xviii, 500.

[4] Karsner, H. T., and Ash, J. E., *loc. cit.*

following study was carried on in conjunction with the general study of the relationship of the two systems.[5]

Dogs were anesthetized and four turnip radish seeds deposited in the right heart by means of a glass cannula inserted through the right jugular vein. The thorax was opened under artificial respiration and the injection of the two arterial systems carried out as in the general study,[6] the same animals being used.

EXPERIMENTS.

Series 1.—Constant pressure of 140 mm. in the aorta, and pressures of 0, 10, 20, 30, 40, 60, and 80 mm. in the pulmonary artery. The type experiment was made with a pressure of 140 mm. in the aorta and 40 mm. in the pulmonary artery. With these pressures the embolic area showed a poor and somewhat irregular injection of the pulmonary vessels and capillaries in contrast to the excellent injection of the normal lung. The bronchial vessels were well injected with the red aortic mass and there was extension to the surrounding alveolar and infundibular capillaries somewhat greater in the embolic area than in the normal lung. With decreases in pressure in the pulmonary artery, the injection of the pulmonary vessels in the embolic areas became less complete. In those cases with pulmonary pressures as low as 10 mm. the extension of the bronchial vessel injection masses in the embolic areas to the capillaries near the bronchi was not more marked than in the normal lung. When, however, the pressure became zero in the pulmonary artery, the extension of the bronchial injection masses to the pulmonary capillaries in the embolic areas became more marked, but never so marked as in the normal lung. With increases in the pulmonary pressures the injection of the embolic areas through the pulmonary artery became more marked and was practically complete at 80 mm. At 60 mm. there was still considerable extension of the bronchial mass to the surrounding capillaries in the embolic area, greater than in the normal lung, but at 80 mm. this extension was not found. Throughout this series the mixture of bronchial mass and pulmonary mass in the bronchial capillaries in the embolic areas remained practically the same.

Series 2.—Constant pressure of 40 mm. in the pulmonary artery and pressures of 0, 40, 60, 100, 140, 160, 180, and 200 mm. in the aorta. The same experiment is regarded as the type in this series as in series 1. With decreases in aortic pressure down to 40 mm. there was progressively less tendency for the aortic injection mass to pass from the bronchial vessels to the alveolar and infundibular areas in the embolic area, and with a pressure of only 40 mm. in the aorta the aortic injection mass was strictly limited to the bronchial vessels. On the other hand, increases in aortic pressure insured greater penetration of the aortic injection mass into the alveolar and infundibular capillaries, so that the embolic

[5] Ghoreyeb, A. A., and Karsner, H. T., *loc. cit.*

[6] Ghoreyeb, A. A., and Karsner, H. T., *loc. cit.*

area showed somewhat more aortic injection mass than pulmonary mass. This extension is more marked in the pulmonary capillaries near the bronchi than in those a little distance removed. Throughout the series the degree of injection through the pulmonary vessels remained the same, not diminishing in extent with the increasing extension from the bronchial vessels. The mixture of the two masses in the bronchial vessels remained practically the same until pressures of 180 and 200 mm. were reached in the aorta, when the amount of pulmonary mass was considerably diminished as compared with that from the aorta.

Series 3.—Constant pressure of zero in the pulmonary artery and pressures of 60 and 140 mm. in the aorta. In all these experiments the embolic area was clearly but not sharply defined from the normal lung. With increases in pressure the embolic area showed progressively better injection, that with a pressure of 180 mm. being almost as complete as in the normal lung injected through the bronchial artery. A striking difference in the injections is the fact that whereas the use of the low pressure left many of the capillaries uninjected, the use of the highest pressure brought about injection of practically all the vessels in the embolic area. In the latter case, however, the injection mass appeared as smaller strands. This condition is due in all probability to low pressure in the embolic area rather than to constriction of the capillaries, because, as has been stated in an earlier publication,[7] the embolic area at the end of one half hour is congested rather than pale, as it would be if vasoconstriction prevailed.

Series 4.—Constant pressure of zero in the aorta and pressures of 40 and 80 mm. in the pulmonary artery. The lower pressure produced very imperfect injection of the embolic area and also of the bronchial vessels. With the higher pressure, however, the injection was almost complete, and was apparently favored by the absence of pressure in the bronchial artery. In the embolic area the bronchial vessels did not show as complete injection as in the normal lung, due to the fact that although the diminished pressure in the embolic area was sufficient for the injection of the pulmonary vessels, it was not sufficiently high to provide for good injection of the bronchials.

SUMMARY.

From series 1 it is concluded that increases in pulmonary pressure improve the circulation in the embolic areas and that decreases in pulmonary pressure limit the circulation in the same areas. In this series the bronchial vessels showed no tendency to take up the circulation in the area of pulmonary embolism until the pulmonary pressure was as low as zero, and then only to a limited degree. It has been shown, however, that in the circulation of an entire lobe the fall to zero pressure in the pulmonary circuit is followed by almost complete taking over of the circulation by the bronchial arterial supply and the most reasonable explanation of why this does not

[7] Karsner, H. T., and Ash, J. E., *loc. cit.*

occur when embolism is present in a smaller branch of the pulmonary is the inference that the physiological anastomosis between the two systems takes place in part before the pulmonary artery breaks up into branches small enough to be occluded by seeds of about three millimeters in diameter.

From series 2 it is concluded that although increases in pressure in the bronchial arteries cause somewhat greater inflow of bronchial injection mass into the embolic area, extremely high pressures are not sufficient to provide for complete circulation in the same district. This fact would tend to support further the belief that the anastomosis between the two vascular systems occurs before the smaller divisions of the pulmonary artery are reached.

From series 3 it is concluded that with a zero pressure in the pulmonary artery there is improved injection of the embolic area through the bronchial artery which is to be expected when it is remembered that zero pressure in the pulmonary artery favors a taking over of the circulation by the bronchial system. What pressure is found in the smaller pulmonary vessels is supplied by the bronchials. It has been shown that the anastomosis between the two systems probably occurs before the branches are reached which would provide lodgment for the turnip radish seeds. The pressure supplied by the bronchials would naturally be less beyond this point and hence the injection would be less complete in the embolic area than in the normal lung. This is shown to be the case by the results of the experiment.

From series 4 it is concluded that the absence of pressure in the bronchial circulation favors a better injection of the embolic area through the pulmonary vessels, which is to be expected when it is recalled that not until zero pressure is reached in the bronchial circuit does the pulmonary artery supply the bronchial vessels with blood.

Throughout the study it was found that whenever the two vascular systems were injected simultaneously the pleural vessels over the embolic area, as well as those over the normal lung, received their supply from the pulmonary vessels. When only one system was used for injection the pleural vessels over the embolic area

showed about the same degree of injection as those of the embolic area itself.

CONCLUSIONS.

The same laws for mixture of the blood of bronchial and pulmonary vessels laid down in connection with the circulation in the lung in general, apply also to the circulation in an area of embolism; *i. e.*, there is no notable mixture of the bloods until the pressure in the one system or the other sinks to zero. Simple embolism of the pulmonary artery results in lowered pressure in the embolic area, the pressure, however, not sinking to zero unless the blood supply of an entire lobe is cut off.

The circulation in the embolic area may be improved by increasing the pressure in either the pulmonary or bronchial arteries, but so long as the two circulations are going on, extremely high pressures are not sufficient to restore the circulation to normal in the area of embolic congestion.

With normal pressures in the two vascular systems, an area of pulmonary embolism involving less than the entire lobe receives its blood supply almost entirely from the rich anastomosis of the pulmonary artery between its own branches, and only when the bronchial pressure is raised to an extremely high point does the blood from this vessel play a notable part in the circulation in the area.

THE PURINES AND PURINE METABOLISM OF SOME TUMORS IN DOMESTIC ANIMALS.*

By ESMOND R. LONG.

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The experiments here reported were performed in connection with another series of similar design, already reported,[1] upon tumors in man. In this publication it was shown that the purines found on hydrolysis of both benign and malignant tumors are the same as those found in normal tissues, and exist in much the same relative proportions. The proportion of the total nitrogen of the tumors constituted by purine nitrogen was found to be less than was to be expected from the histological evidence of the amount of nuclear material present. The purine enzymes of the tumors seemed to be the same as those of the normal tissue from which the tumors were derived, but secondary tumors in the liver did not acquire the characteristic purine enzyme, xanthine oxidase, of that organ in man.

As the distribution of purine enzymes is not the same in the organs of different species, it was thought advisable in connection with the experiments upon human tumors, to examine animal tumors for comparison. It will be recalled that the following enzymes are concerned in nucleoprotein metabolism: nucleases, which split nucleic acid from its protein combination, and break the acid down into its constituents, liberating the two purines, guanine and adenine; guanase, which converts guanine into xanthine; adenase, which converts adenine into hypoxanthine; xanthine oxidase, which converts xanthine, and possibly hypoxanthine, into uric acid; and uricase, which breaks up the uric acid into non-purine substances.

It has recently been shown by Amberg and Jones[2] that besides these there are enzymes capable of splitting up nucleic acid in such a way that xanthine and hypoxanthine are formed from the guanine and adenine radicals, respectively, of nucleic acid, but by way of

* Received for publication, June 2, 1913.

[1] Wells, H. G., and Long, E. R., *Ztschr. f. Krebsforsch.*, 1913, xii, 598.

[2] Amberg, S., and Jones, W., *Ztschr. f. physiol. Chem.*, 1911, lxxiii, 407.

other intermediate products than guanine and adenine; in the case of xanthine, from guanosine and xanthosine, and, in the case of hypoxanthine, from adenosine and inosine.[3] The enzymes which act upon these substances are guanosine deaminase, which converts guanosine, a product of the action of a nuclease, into xanthosine; and xanthosine hydrolase, which converts xanthosine into xanthine; adenosine deaminase, which converts adenosine, produced by the action of a nuclease, into inosine; and inosine hydrolase, which converts inosine into hypoxanthine. The distribution of these enzymes appears to be very extensive.

Nucleases appear to be present in all tissues. Proof of their presence in tumor tissue has been given by Goodman,[4] who found in carcinomata of the breast a nuclease which split up thymus nucleic acid. Guanase is widely distributed in the organs of different species. The pig only has difficulty in disposing of its guanine, guanine gout being a characteristic disease of this animal. Adenase is much less widely distributed, its occurrence in human tissues being disputed. The oxidizing enzyme, xanthine oxidase, is usually found in the liver, and occasionally in other organs. Uricase is found in the kidney or liver of all mammals investigated, except man.[5] The small amount of investigation of these enzymes in tumor tissue has already been summarized.[6]

The method of analysis used in the following experiments is that of Krüger and Solomon, in which the purines are precipitated with copper sulphate and sodium bisulphite and then isolated, guanine and xanthine as the free bases, and adenine and hypoxanthine respectively as the picrate and the silver nitrate. In the enzyme experiments the purine under investigation was added to an emulsion of 50 to 100 grams of the fresh tumor tissue in 500 cubic centimeters of water, rather than to a stained extract of the tissue, and kept at 38° C. This method is disadvantageous in that thorough washing is nearly impossible in the filtration from tissue prelimi-

[3] Levene, P. A., and Jacobs, W. A., *Ber. d. deutsch. chem. Gesellsch.*, 1908, xli, pt. 2, 2703; 1909, xlii, pt. 1, 335, pt. 2, 2469; 1911, xliv, pt. 1, 746.

[4] Goodman, E. H., *Jour. Exper. Med.*, 1912, xv, 477.

[5] For a detailed review of this subject the reader is referred to Wells, H. G., *Jour. Biol. Chem.*, 1909–10, vii, 171.

[6] Wells, H. G., and Long, E. R., *loc. cit.*

nary to the separation of the purines, some of the purines being held mechanically in the pieces of tissue. On the other hand, it possesses an advantage in that the large amount of protein protects the purines from harmful change in the reaction of the medium during autolysis, and also probably furnishes a higher concentration of enzyme than is obtainable by the extraction method. Toluene was used as the antiseptic.

Repeated experience has shown that under the conditions of the following experiments added purines in the amounts used are completely converted in the presence of the specific enzymes, in the time of incubation allowed, which was in all cases about fourteen days. Therefore, the presence of an enzyme is not considered conclusively demonstrated unless the added purine cannot be recovered at all.

PURINES OF ANIMAL TUMORS.

Tumor 1.—This was a large tumor of the testicle in a bovine cryptorchid. The microscopic appearance was that of an endothelial sarcoma of the type frequently seen in testicle tumors in man, with an abundant stroma and very little necrosis. 1,315 gm. were hydrolyzed with dilute sulphuric acid until biuret-free. The solution was neutralized with sodium hydroxide, made acid with acetic acid, and filtered from melanin. The total nitrogen, other than melanin nitrogen, the purine nitrogen, and the amounts of the individual purines, were determined and found to be as follows:

Total nitrogen per 1,000 gm. of fresh material	21.63
Per cent. of total nitrogen as purine nitrogen	1.61

Purines Recovered.

Guanine	0.416 gm., or 46 per cent.
Xanthine	0.150 gm., or 17 per cent.
Adenine	0.229 gm., or 25 per cent.
Hypoxanthine	0.106 gm., or 12 per cent.

The bulk of the recovered purines thus consisted of the amino purines, guanine and adenine, as usually found in the analysis of fresh tissue, but there was a considerable amount of xanthine and hypoxanthine.

Tumor 2.—A portion of a large tumor in the kidney of a pig was stripped free from the kidney tissue and used for analysis. The microscopic findings in the sections were as follows: The tumor is of the type of so called embryonal adenoma or adenosarcoma of the kidney. It is chiefly adenomatous with an abundant, highly cellular stroma. In places the stroma approaches the sarcomatous type, but in general it resembles young fibrous tissue. Tumors of this type in swine have been described in detail by Day.[7] 1,674 gm. of this tumor

[7] Day, L. E., *Twenty-Fourth Annual Report of the Bureau of Animal Industry*, 1907, 247.

were hydrolyzed with 7 per cent. sulphuric acid until biuret-free, and the hydrolyzed material was treated as described above. The results of analysis were:

Total nitrogen per 1,000 gm. of fresh material 23.45
Per cent. of total nitrogen as purine nitrogen 4.19

As the solution resulting from the hydrolysis was large, it was divided into two portions for the separation of the purines. An unexpected result was obtained. The figures for the duplicates were as follows:

	A.	B.	Total.
Guanine	0.610 gm.	0.588 gm.	1.198 gm., or 37 per cent.
Xanthine	0.390 gm.	0.436 gm.	0.826 gm., or 25 per cent.
Adenine	0	0	0
Hypoxanthine	0.602 gm.	0.610 gm.	1.212 gm., or 38 per cent.

Analysis[8] of the purified products for nitrogen by the Kjeldahl method gave:

Xanthine ..... 36.89 per cent.
Calculated ..... 36.84 per cent.
Hypoxanthine as anhydrous hydrochloride ..... 32.36 per cent.
Calculated ..... 32.46 per cent.

Apparently the nucleic acid or acids of this tumor contained no adenine. The presence of large quantities of xanthine and hypoxanthine, which are ordinarily produced secondarily from guanine and adenine by deaminization, is hard to account for in a fresh tissue showing no evidence of autolysis. It is possible that several of the so called mononucleic acids, *i. e.*, nucleic acids containing only one purine base, were present. We have in inosinic acid a nucleic acid containing hypoxanthine as its only purine constituent. According to the results of several observers[9] inosinic acid yields hypoxanthine upon hydrolysis according to the following equation:

$$C_{10}H_{13}N_4O_8P + 2H_2O \rightarrow C_5H_5N_4O + H_3PO_4 + C_5H_{10}O_5.$$

In a similar way guanylic acid contains only guanine.

It was thought that determination of the purine content of normal kidney tissue would throw some light upon the situation. Therefore a similar analysis of some normal pigs' kidneys was made, with the following results, 905 grams of ground tissue stripped from pelvic fat being used:

[8] In order to avoid the possibility of error in the analysis of the hypoxanthine fraction, in which adenine might have been carried along through failure of precipitation with picric acid, and subsequently deaminized by traces of nitrous acid in the nitric acid used, in all operations involving a use of the latter a few crystals of urea were added to destroy the nitrous acid.

[9] Neuberg, C., and Brahn, B., *Biochem. Ztschr.*, 1907, v, 438; *Ber. d. deutsch. chem. Gesellsch.*, 1908, xli, pt. 3, 3376. Levene, P. A., and Jacobs, W. A., *ibid.*, pt. 2, 2703.

Total nitrogen per 1,000 gm. of fresh material	25.01
Per cent. of total nitrogen as purine nitrogen	3.27

Purines Recovered.

Guanine	0.524 gm., or 48 per cent.
Xanthine	trace
Adenine	0.368 gm., or 33 per cent.
Hypoxanthine	0.207 gm., or 19 per cent.

The melting point of the adenine picrate was 285° C. This result is in accordance with the usual findings of the purine base content of fresh tissues. Xanthine is absent, while hypoxanthine is present in relatively small amount. Some seems to be found in all tissues, the preformed hypoxanthine mentioned by Walter Jones and his co-workers.

Other similar tumors of pigs' kidneys were secured with the idea of ascertaining if the observed peculiar features were at all constant.

Tumor 3.—In some sections, on microscopic examination, no vestige of the original kidney tissue is seen. In others the tubules are widely separated by infiltration of small round cells. In many respects the structure resembles that of the leukemic kidney, but there is no marked excess of leucocytes in the blood vessels outside the tumor. There is some hemorrhage, but no necrosis. Diagnosis: small round cell sarcoma.

950 gm. of this tumor were hydrolyzed and analyzed as usual.

Total nitrogen per 1,000 gm. of fresh material	23.62
Per cent. of total nitrogen as purine nitrogen	5.32

Purines Recovered.

Guanine	1.020 gm., or 44 per cent.
Xanthine	trace
Adenine	0.931 gm., or 41 per cent.
Hypoxanthine	0.340 gm., or 15 per cent.

The melting point of the adenine picrate was 283° C. Here, as in the case of the normal kidney, the result is quite different from that obtained with the adenosarcoma. Guanine and adenine constitute 80 to 85 per cent. of the purified purines, the former being present in slightly greater proportion, as is generally found. The individual purines are found in relative proportions not far removed from those found in the normal kidney.

Tumor 4.—Microscopically the tumor consists of irregular but well differentiated tubules with areas with a tendency to papillary formation, the cells being low columnar in type. The stroma in the immediate proximity of the tubules is of the large spindle cell type. Between the groups of tubules and surrounding cellular stroma is another stroma of more mature connective tissue giving an alveolar appearance to the entire growth. Diagnosis: embryonal adenosarcoma.

552 gm. of this tumor were hydrolyzed, and a portion of the solution was

analyzed as usual, with the following results. The figures given are corrected for the entire amount.

Total nitrogen per 1,000 gm. of fresh material	20.45
Per cent. of total nitrogen as purine nitrogen	5.9

Purines Recovered.

Guanine	0.597 gm.,	or 41 per cent.
Xanthine	0.059 gm.,	or 4 per cent.
Adenine	0.614 gm.,	or 42 per cent.
Hypoxanthine	0.119 gm.,	or 13 per cent.

The identity of the adenine picrate was made certain by its melting point, 285° C. So here again the amino purines predominate, and adenine makes up the usual share of the purine content.

Thus of these several tumors, with the exception of one, all contained guanine and adenine in predominating amount, 70 to 85 per cent., xanthine only in small amount, and hypoxanthine in larger amounts. The variation from the nuclein composition of the tissue from which the tumor was derived was not great in the case of tumors of the kidney, nor was the relative distribution of the purines different from that usually observed in fresh tissue. Neuberg[10] has found that the nucleoprotein of tumor tissue does not differ in quantitative composition from that of tissues in general. No safe general conclusions can be drawn with regard to the first adenosarcoma studied, because of inability to repeat the result, but apparently its nuclein material was devoid of adenine and contained large amounts of xanthine and hypoxanthine.

The histological appearance of the tumors of the kidney corresponded well with the increase in nuclein material in the tumors over that found in the normal tissue, as found by analysis. The per cent. of total nitrogen present as purine nitrogen in the kidney was 3.27 per cent. In the first adenosarcoma, which histologically showed more nuclear material, it was 4.19 per cent., while in the small round cell sarcoma, which was much more cellular than the adenosarcoma, it was 5.32 per cent.

PURINE ENZYMES OF TUMORS.

Experiment 1.—This was the endothelial sarcoma, No. 1, described above. In all the following experiments an emulsion containing a weighed amount of

[10] Neuberg, C., *Ztschr. f. Krebsforsch.*, 1910, x, 55.

tissue with weighed amounts of the purines in toluene water was kept in an air-tight bottle at 38° C. for fourteen days with occasional shaking. The hydrochloride of guanine and sulphate of adenine were used and the purines were recovered as free guanine and xanthine, adenine picrate, and hypoxanthine silver nitrate. The figures given are calculated for the free purines; this custom will be followed for brevity's sake in the tables that follow. The analytical data for this experiment were as follows:

Tissue	Guanine added,	Guanine recovered,	Xanthine recovered,
100 gm.	0.157 gm.	trace	0.096 gm.
Tissue	Adenine added,	Adenine recovered,	Hypoxanthine recovered,
100 gm.	0.191 gm.	0.110 gm.	0.045 gm.

The purines chiefly recovered were thus xanthine and adenine. A small amount of hypoxanthine was also recovered. It will be seen in the analyses that this is regularly formed in the autolysis of tissues, even in the absence of adenase. According to Amberg and Jones,[11] hypoxanthine may be derived from the adenine radicle of nucleic acid in tissue autolysis without passing through the adenine stage, adenosine and inosine being the intermediate products, which are broken up by specific enzymes. Therefore, it may be concluded from the above data that guanase was present, as guanine could not be recovered, its place being taken by xanthine, and that adenase was absent, the added adenine being recovered.

A separate test was made for the presence of the oxidizing enzyme xanthine oxidase, by means of which xanthine is converted into uric acid. 100 gm. of ground tissue were placed in toluene water containing 0.248 gm. of xanthine and the mixture was kept at 38° C. for forty-four hours while a current of warm air was sucked through. The results were as follows:

Tissue	Xanthine added,	Xanthine recovered,	Uric acid recovered,
100 gm.	0.248 gm.	0.204 gm.	0

The Horbaczewski test carried out upon the entire amount of recovered purine demonstrated the absence of uric acid. Evidently this tissue did not possess xanthine oxidase.

Through Dr. Day it was possible to secure for comparison some cryptorchid testicles. The microscopic appearance of these organs was that of undescended testicle with the usual undeveloped condition of the germinal epithelium and hyperplasia of the interstitial elements. Tests for the same enzymes were made with the following results:

Tissue	Guanine added,	Guanine recovered,	Xanthine recovered,
45 gm.	0.157 gm.	0	0.113 gm.

[11] Amberg, S., and Jones, W., *Ztschr. f. physiol. Chem., loc. cit.*

Tissue	Adenine added,	Adenine recovered,	Hypoxanthine recovered,
45 gm.	0.121 gm.	0.091 gm.	0.045 gm.

Again guanine has disappeared and adenine remained; guanase may be considered present and adenase absent. The absence of xanthine oxidase was demonstrated by the inability of the tissue to form uric acid from xanthine.

Tissue	Xanthine added,	Xanthine recovered,	Uric acid recovered,
45 gm.	0.224 gm.	0.160 gm.	0

Thus the enzyme content of the tumor and normal tissue from which it was derived, was, as respects the enzymes studied, exactly the same, guanase being present, and adenase and xanthine oxidase absent, in both.

Experiment 2.—This was a tumor from the ovary of a cow, weighing 1,250 gm. Microscopic examination showed it to be an endothelioma with very little stroma. The tests for the presence of guanase, adenase, and xanthine oxidase were made as above. The analyses resulted as follows:

Tissue	Guanine added,	Guanine recovered,	Xanthine recovered,
100 gm.	0.170 gm.	0	0.128 gm.

Tissue	Adenine added,	Adenine recovered,	Hypoxanthine recovered,
100 gm.	0.142 gm.	0.083 gm.	0.038 gm.

With Warm Air Current for Forty-Eight Hours.

Tissue	Xanthine added,	Xanthine recovered,	Uric acid recovered,
100 gm.	0.222 gm.	0.141 gm.	0

The recovery of xanthine was low because of the great difficulty in filtering and washing in consequence of the presence of much mucilaginous material in the tumor. However, as no uric acid was formed,—the murexide test carried out on the recovered purines was negative,—it may be assumed that xanthine oxidase was absent. The tissue apparently contained guanase and lacked adenase, xanthine and adenine being the recovered purines. Some normal cows' ovaries were obtained for comparison. The results of analyses after fourteen days' incubation were as follows:

Tissue	Guanine added,	Guanine recovered,	Xanthine recovered,
100 gm.	0.157 gm.	0	0.182 gm.

Tissue	Adenine added,	Adenine recovered,	Hypoxanthine recovered,
100 gm.	0.121 gm.	0.065 gm.	0.009 gm.

Tissue	Xanthine added,	Xanthine recovered,	Uric acid recovered,
100 gm.	0.224 gm.	0.209 gm.	0

Unfortunately, a sample of adenine hydrochloride from an old stock had to be used for this experiment. It was later found to be contaminated with inorganic material. This accounts for the low recovery of adenine. However, from the fact that 0.065 of a gram of adenine was recovered while only a trace of hypoxanthine was found it may safely be concluded that adenase was not present. Guanase was clearly present and xanthine oxidase absent. So here again the tumor tissue contained exactly the same enzymes, as far as tested, as normal ovarian tissue from which the tumor was derived. It will be noticed that the enzyme content of the reproductive glands in both sexes of the bovine, as far as guanase, adenase, and xanthine oxidase are concerned, is the same.

Experiment 3.—This was a tumor mass in the liver of a sheep, weighing 1,200 gms. Only a small amount of liver tissue remained. The microscopic appearance was as follows: It has a carcinomatous appearance with a tendency to papillary formation. The cells have a deep staining cytoplasm with much vacuolization, so that the tumor bears strong resemblance to a hypernephroma, but the appearance is not at all incompatible with the findings in malignant adenoma of the liver, especially in respect to the structure of the cytoplasm.

The results for the tumor were as follows:

Tissue	Guanine added,	Guanine recovered,	Xanthine recovered,
100 gm.	0.156 gm.	0	0.166 gm.
Tissue	Adenine added,	Adenine recovered,	Hypoxanthine recovered,
100 gm.	0.151 gm.	0	0.113 + gm.

An accident occurred with the hypoxanthine fraction, reducing the recovery by about a fifth.

A control containing the same ingredients in the same amount was boiled one hour before incubation. The results of the analysis were as follows:

Tissue	Guanine added,	Guanine recovered,	Xanthine recovered,
100 gm.	0.156 gm.	0.095 gm.	0.013 gm.
Tissue	Adenine added,	Adenine recovered,	Hypoxanthine recovered,
100 gm.	0.151 gm.	0.112 gm.	0.019 gm.

From these data it is clear that both guanase and adenase were present in the tumor tissue, xanthine and hypoxanthine and no guanine or adenine being recovered after treatment of guanine and adenine with the fresh tissue, while guanine and adenine were recovered in quantity from the boiled control. The traces of xanthine and hypoxanthine found in the analysis of the boiled control were probably present in the tissue before boiling.

Xanthine oxidase was also sought in this tissue:

Tissue	Xanthine added,	Xanthine recovered,	Uric acid recovered,
100 gm.	0.224 gm.	0.301 gm.	0
	Boiled Control.		
100 gm.	0.224 gm.	0.189 gm.	0

The murexide test proved the absence of uric acid from the purine recovered from the treatment with the fresh tissue. The large amount of xanthine recovered is probably to be explained as the result of the active autolysis in a tissue with high nuclear content, from the guanine of its nucleic acid.

The liver tissue from which the tumor had grown was also examined, with the following result:

Tissue	Xanthine added,	Xanthine recovered,	Uric acid recovered,
100 gm.	0.224 gm.	0	0
	Boiled Control.		
100 gm.	0.224 gm.	0.182 gm.	0

Xanthine was recovered almost quantitatively from the boiled control, while neither xanthine nor uric acid could be recovered from the experiment with the fresh tissue. The addition of copper sulphate and sodium bisulphite to the filtrate from the tissue after incubation failed to produce any precipitate whatever, showing the complete absence of purines. In other words the presence of the enzyme which destroys uric acid as well as that of the enzyme which forms it from xanthine, seems here to be demonstrated. This is in agreement with the results of Battelli and Stern,[12] who found uricase in the sheep's liver.

The same liver was examined for the presence of guanase and adenase. Guanase was found, but for some unknown reason adenase was not. This was peculiar because a second trial made upon a normal liver demonstrated its presence perfectly.

This tumor then contained both guanase and adenase, as does normal sheep liver tissue, but differs from the latter in not containing xanthine oxidase. It has already been shown[13] that secondary tumors growing in the human liver do not acquire the enzyme xanthine oxidase. This fact, and the histological resemblance of the tumor to a hypernephroma, suggested the possibility of relationship of this tumor with the kidney or adrenal. Accordingly these organs from normal sheep were examined for the presence of xanthine oxidase and adenase. Both enzymes, however, were found

[12] Battelli, F., and Stern, L., *Biochem. Ztschr.*, 1909, xix, 219.

[13] Wells, H. G., and Long, E. R., *loc. cit.*

to be absent from both organs. As experience with other tumors has regularly shown that where adenase is absent from the normal tissue, it is also not found in the tumor growth, it is probable that the resemblance to a hypernephroma was purely morphological, and the tumor was a true liver carcinoma, which had retained the two deaminizing enzymes but had lost the oxidizing one.

It is interesting to note that the enzyme present in the liver and lacking in the tumor, xanthine oxidase, is, next to uricase, the last of the purine enzymes to develop in the embryo. Mendel and Mitchell,[14] and Jones and Austrian[15] have shown that xanthine oxidase is not present in the liver of the pig of 200 mm. or under, but appears about the time of birth. Wells and Corper[16] found that xanthine oxidase, present in the liver of the full term human infant, is not contained in the liver of the six months' fetus. It is probable that a similar condition obtains in other animals, for example, the sheep. The finding, therefore, of this enzyme, which ordinarily appears late in the course of the development of an animal, in the normal liver, and the failure to demonstrate it in the tumor of the liver, are evidence, on chemical grounds, of the embryonal nature of this malignant tumor, corroborating the general assumption of embryonal nature of these tumors made on a histological basis. Also, the finding of a difference between the enzyme content of these tumors and that of kidney and adrenal tissue, suggests that chemical studies may be of value in clearing up the disputed question of origin of these tumors which histologically resemble hypernephromas.

An exactly similar tumor could not be obtained. The nearest approach to it which macroscopically bore resemblance to tumors of the renal type is the next one to be described.

Experiment 4.—This was a smaller tumor in a sheep's liver. Microscopically the tumor is a tubular carcinoma, in structure resembling the so called bile duct carcinomata. A large proportion is necrotic; many areas of calcification are present. The tumor tissue consists of columns of epithelial cells with some tendency to the formation of tubules, between which is a moderate amount of stroma. The tumor cells have small, deeply staining, solid nuclei with cytoplasm staining heavily with eosin, like the cytoplasm of liver cells, with no considerable tendency to vacuolization.

[14] Mendel, L. B., and Mitchell, P. H., *Am. Jour. Physiol,* 1907–08, xx, 97.
[15] Jones, W., and Austrian, C. R., *Jour. Biol. Chem.,* 1907, iii, 227.
[16] Wells, H. G., and Corper, H. J., *Jour. Biol. Chem.,* 1909, vi, 469.

The analytical data are as follows, adenase only being sought for comparison with kidney and adrenal tissue:

Tissue	Adenine added,	Adenine recovered,	Hypoxanthine recovered,
50 gm.	0.134 gm.	0	0.090 gm.
	Boiled Control.		
50 gm.	0.134 gm.	0.130 gm.	0.012 gm.

The hypoxanthine fraction in the experiment with the fresh tissue was treated with urea to prevent deaminization of any adenine possibly present, in the use of nitric acid, and recrystallized as the hydrochloride. The latter was made anhydrous and subjected to analysis:

Analysis ..... 32.34 per cent. nitrogen.
Calculated for $C_5H_5N_4OHCl$ ..... 32.47 per cent. nitrogen.

Adenase was clearly present in this tumor, for hypoxanthine was recovered in place of adenine in the experiment, while adenine was recovered quantitatively from the boiled control. The relationship with the liver, rather than with the kidney or adrenal, is thus established.

As the pig differs from all other animals in its difficulty in disposing of guanine, some pig tumors were studied.

Experiment 5.—This tumor was in the kidney of a pig. It is the same as tumor 2, described above. Xanthine oxidase, adenase, and guanase were tested for as usual. In the xanthine oxidase experiment a fault in the air current permitted the suction back of part of the mixture from the boiled control into the emulsion of fresh tissue, rendering a composite analysis necessary. Xanthine was recovered in quantity, and no uric acid:

Tissue	Xanthine added,	Xanthine recovered,	Uric acid recovered,
100 gm.	0.230 gm.	0.296 gm.	0

The murexide test applied to the recovered xanthine was negative. Xanthine oxidase was therefore not present.

The experiments with guanase and adenine resulted as follows:

Tissue	Guanine added,	Guanine recovered,	Xanthine recovered,
50 gm.	0.163 gm.	0.107 gm.	0.004 gm.
Tissue	Adenine added,	Adenine recovered,	Hypoxanthine recovered,
50 gm.	0.128 gm.	0.065 gm.	0.080 gm.
	Boiled Control.		
50 gm.	0.163 gm.	0.075 gm.	0.004 gm.
50 gm.	0.128 gm.	0.081 gm.	0.050 gm.

For some reason the losses in analysis were unusually large with this tumor, but apparently guanase is not present, guanine being recovered from the treatment with fresh tissue in even greater quantity than from the control, while only traces of xanthine were found in either. The results with the adenine are less sharp. At least it may be said that the power of destroying adenine, if it is a property of the renal tissue, is not great, for over half the adenine was recovered. A large part of the high hypoxanthine recovery of course comes from the tissue, which was rich in hypoxanthine (page 515).

The effect of normal kidney tissue upon xanthine, guanine, and adenine, was also studied, and xanthine oxidase, guanase, and adenase were all found to be absent. Of the tissues examined in these experiments pig kidney and tumors derived from it were the only tissues which did not contain guanase.

Experiment 6.—The sarcoma of the pig's kidney (page 516) was also studied with respect to its enzyme content. The results were as follows:

Tissue	Guanine added,	Guanine recovered,	Xanthine recovered,
50 gm.	0.165 gm.	0.176 gm.	0.024 gm.
Tissue	Adenine added,	Adenine recovered,	Hypoxanthine recovered,
50 gm.	0.143 gm.	0.096 gm.	0.060 gm.
		Boiled Control.	
50 gm.	0.165 gm.	0.122 gm.	0.015 gm.
50 gm.	0.143 gm.	0.138 gm.	0.021 gm.

These results are comparable to those obtained with the other tumor in the pig kidney. Clearly guanase is absent and adenase is apparently also absent. It will be noted that in both cases the recovery of guanine was greater in the experiment with the fresh tissue than in that with the boiled control. A similar experience was met with in the examination of the normal pig's kidney. This feature is to be explained, probably, as the result of autolysis of tissue with liberation of guanine, and inability to destroy the guanine in the absence of guanase. Of course no such autolysis would take place in the boiled tissue. The increased yield of hypoxanthine over that found in the control in the absence of adenase is possibly the result of decomposition of the adenine radicle of the nucleic acid by another route, namely, by way of adenosine and inosine,[17] adenosine deaminase and inosine hydrolase being the enzymes concerned. We have, however, no experimental proof of this for the present case.

These results seem to show that the pig's kidney is poorly pro-

[17] Amberg, S., and Jones, W., *loc. cit.*

vided with purine enzymes, and that the same deficiency is to be noted in tumors derived from the kidney.

The distribution of purine enzymes as determined in this investigation, may be summarized briefly as follows, + meaning present, and — absent.

Tissue.	Guanase.	Adenase.	Xanthine oxidase.
Bovine			
Cryptorchid	+	—	—
Endothelial sarcoma from cryptorchid	+	—	—
Ovary	+	—	—
Endothelioma of ovary	+	—	—
Sheep			
Liver[18]	+	+	+
Kidney		—	—
Adrenal		—	—
Carcinomata of liver	+	+	—
Pig			
Kidney	—	—	—
Adenosarcoma of kidney	—	—	—
Sarcoma of kidney	—	—	—

SUMMARY.

The purines of tumors are generally the same as those of the normal tissue, chiefly guanine and adenine, little more than traces of xanthine, and relatively small amounts of hypoxanthine being found. No uric acid was found in the tumors of this series.

In one tumor, an embryonal adenosarcoma in a pig's kidney, a large amount of hypoxanthine, and no adenine was found, while the yield of xanthine was also unusually high. This result cannot be explained at present on experimental grounds, but evidently a nucleic acid complex containing no adenine was encountered. Possibly hypoxanthine was present in inosinic acid.

The purine enzymes are generally the same in tumors as in the normal tissue from which the tumors are derived. This fact sometimes makes it possible to learn by chemical study from what tissue a given kind of tumor is derived, when the microscopic evidence is inconclusive.

Although the liver of the sheep contains the enzyme xanthine

[18] Contains uricase also.

oxidase, it may be absent from primary tumors in the sheep liver, as it has also been shown to be from secondary tumors in the human liver. As xanthine oxidase is one of the last of the purine enzymes to develop in the embryonal organism, its absence in a malignant tumor containing otherwise the same purine enzymes as the tissue from which it is derived, is evidence of the embryonal character of the tumor tissue.

I am indebted to Dr. H. G. Wells for many suggestions and for the diagnosis of the pathological material, and to Dr. L. E. Day, of the U. S. Bureau of Animal Industry of the Chicago Stock Yards, for furnishing the material which made this investigation possible.

ON THE HEMOLYTIC PROPERTIES OF FATTY ACIDS AND THEIR RELATION TO THE CAUSATION OF TOXIC HEMOLYSIS AND PERNICIOUS ANEMIA.*

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In the many attempts that have been made to interpret the phenomena of pernicious anemia by the supposition that a powerful hemolytic agent is at large in the body, the fact that substances soluble in alcohol or ether may have strong hemolytic properties has been much insisted upon. More especially attention has been directed to fatty acids and their alkaline salts, and since the work of Faust and Tallquist (1), in which the anemia caused by *Bothriocephalus latus* was ascribed to the unsaturated acid, oleic acid, which the worm contains, there has been a tendency to regard particularly the unsaturated fatty acids as playing possibly an important part in other forms of anemia. The marked difference described by Faust and Tallquist between the hemolytic properties of oleic acid and its salts on the one hand, and stearic acid and its salts on the other, which was also observed in the comparison of other pairs of acids standing to each other in the same relation as these two, seemed to indicate that the lytic power of oleic acid might reside in the one chemical point of difference between it and stearic acid; namely, the pair of doubly united carbon atoms present in its molecule.

In recent years we have come to recognize that oleic acid is not the only unsaturated fatty acid that occurs in the body. We have clear evidence that in animal cells, with probably the single exception of the adipose connective tissue corpuscles in which the crude food fat is stored after absorption if not needed for immediate use, there are considerable quantities of fatty acids in which more than one such pair of doubly united carbon atoms occur in each molecule. If, therefore, in oleic acid the single pair of unsaturated atoms gives

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rise to such remarkable properties in relation to red blood corpuscles, it seemed possible that the hemolytic properties of the fatty acids more unsaturated than oleic acid, with two, three, or even four, such pairs of doubly united carbon atoms, might be far greater still. And if this were so, a new direction might be given to attempts such as those of Joannovics and Pick (2) to look for the cause of toxic anemias in the fatty products liberated from cells destroyed by the action of a toxic principle.

In order to test this theory it was necessary in the first instance to fix some standard of hemolytic power with which to compare the properties of any substance to be investigated. For this purpose 0.2539 of a gram of oleic acid (Schuchardt's acidum oleinicum purissimum), which was found to have almost the correct molecular weight, 285, and almost the theoretical iodine value (calculated 90.0; observed 87.5), was dissolved in 100 cubic centimeters of methyl alcohol and neutralized with the calculated amount of standard soda solution; and immediately before each experiment one cubic centimeter of this solution was diluted with normal saline and the resulting emulsion used. In all the experiments the same amount of twice washed red blood corpuscles was used, 0.5 of a cubic centimeter of a 5 per cent. suspension; except where expressly mentioned the corpuscles were those of the sheep; and the volume was made up with 0.9 per cent. salt solution in every case to two cubic centimeters. The tubes were shaken, kept at 37° C. for two hours, and the results read then and again after standing for twenty-four hours at room temperature. Table I shows the result on one of many occasions on which the amount of the sodium salt of this oleic acid that was necessary to give complete hemolysis was determined and found to be the same.

TABLE I.

Sodium Oleate in Methyl Alcohol (1 c.c. = 2.75 mg.) Diluted 1 to 20 with Normal Saline (1 c.c. = 0.137 mg.).

Tube.	Lytic agent.	Result.
1	0.1 c.c.	Partial.
2	0.2 c.c. = 0.027 mg.	Partial, almost complete.
3	0.3 c.c. = 0.041 mg.	Complete.
4	0.4 c.c.	Complete.

In order to compare these results with those obtained by other workers, and also with some of those to be described in which human blood corpuscles were employed, on more than one occasion the effect of the same preparation on the blood of other species of animals was determined, and from the results given in table II it is apparent that no difference could be detected in the amounts necessary for complete hemolysis of equal quantities of corpuscles from the sheep, rabbit, ox, dog, and man, for all of which species the minimum hemolytic dose of sodium oleate is about 0.03 of a milligram for 0.5 of a cubic centimeter of a 5 per cent. suspension of corpuscles.

TABLE II.

Sodium Oleate in Methyl Alcohol (1 c.c. = 2.75 mg.) Diluted 1 to 80 with Normal Saline (1 c.c. = 0.034 mg.).

Tube	Lytic agent.	Result.				
		Dog.	Rabbit.	Sheep.	Ox.	Man.
1	0.3 c.c.	Trace	Partial	Trace	Trace	Partial.
2	0.4 c.c.	Partial	Partial	Trace	Trace	Partial.
3	0.8 c.c. = 0.027 mg.	Complete	Complete	Complete	Complete	Complete.
4	1.2 c.c. = 0.039 mg.	Complete	Complete	Complete	Complete	Complete.

We can now compare with this result those obtained by others. Noguchi determined the minimum lytic dose of sodium oleate for 2 c.c. of a 5 per cent. suspension of ox corpuscles to be 0.07 mg.; for the quantity of sheep's corpuscles, 0.5 c.c., used in the experiments here reported, the dose according to Noguchi (3) would be therefore 0.018 mg. Meyerstein (4), who also made careful determinations of the amount of a 0.05 per cent. sodium oleate solution necessary completely to hemolyze 1 c.c. of a 5 per cent. suspension of sheep's corpuscles gives as his result 0.1 c.c., or 0.05 mg.; for 0.5 c.c., therefore, 0.025 mg. Finally from Lamar's (5) results the lytic dose of the same substance for the same amount of corpuscles may be calculated and with the same result. There is thus a fair agreement between these sets of observations.

Noguchi	0.018 mg.
Meyerstein	0.025 mg.
Lamar	0.025 mg.
McPhedran	0.027 mg.

It is therefore clear that the standard for hemolytic activity here adopted is fairly well defined, that between 0.02 and 0.03 of a milligram of sodium oleate will completely lake 0.5 of a cubic centimeter of a 5 per cent. suspension of red blood corpuscles in the hands of

different workers; or, in other words, one milligram is the lytic dose for one cubic centimeter of corpuscles of the rabbit, sheep, ox, dog, or man. When, therefore, a substance is spoken of as having intense or strong or weak hemolytic properties, which is commonly done without reference to any definitely fixed scale or standard, it would be far better if a simple comparison of the minimum hemolytic dose of the substance in question with that of sodium oleate were substituted for often misleading epithets used in designating its activity.

In the tables that follow the results are given of experiments in which a number of fatty acids have been tested out in this way.

Palmitic acid (Schuchardt's purissimum) (table III), as an example of a saturated fatty acid, and probably the one which is most abundant in food, was converted into sodium salt, and a solution in methyl alcohol of which one cubic centimeter contained 2.6 milligrams was prepared. Of this one cubic centimeter diluted ten times with warmed normal saline gave an emulsion with which complete hemolysis was not obtained with the largest quantity used. The minimum hemolytic dose of sodium palmitate is therefore more than 0.4 of a milligram, or more than twelve times as great as that of sodium oleate.

TABLE III.

Sodium Palmitate in Methyl Alcohol (1 c.c. = 2.6 mg.) Diluted 1 to 10 with Normal Saline.

Tube.	Lytic agent.	Result.
1	0.1 c.c.	Trace.
2	0.2 c.c.	Trace.
3	0.4 c.c.	Partial.
4	0.8 c.c.	Partial.
5	0.6 c.c. = 0.42 mg.	Partial.

TABLE IV.

Sodium Erucate in Methyl Alcohol (1 c.c. = 3.32 mg.) Diluted 1 to 10 with Normal Saline (1 c.c. = 0.33 mg.).

Tube.	Lytic agent.	Result.
1	0.1 c.c. = 0.033 mg.	Partial.
2	0.2 c.c. = 0.066 mg.	Complete.
3	0.25 c.c.	Complete.
4	0.3 c.c.	Complete.

TABLE V.

Sodium Linoleate in Methyl Alcohol (1 c.c. = 2.5 mg.) Diluted 1 to 80 with Normal Saline (1 c.c. = 0.03 mg.).

Tube.	Lytic agent.	Result.
1	0.2 c.c.	Partial.
2	0.4 c.c.	Partial.
3	0.8 c.c. = 0.025 mg.	Partial.
4	1.2 c.c. = 0.037 mg.	Complete.

Erucic acid (table IV), a commercial preparation (Schuchardt's), which was found to have the molecular weight 338 and iodine value 61.8 (calculated 340 and 74.6, respectively), was used in a similar way. The minimum lytic dose is about twice that of sodium oleate.

Linoleic acid is an acid that is more unsaturated than oleic acid. There being two pairs of doubly united carbon atoms in the molecule it was conceivable that the hemolytic power might be greater than that of oleic acid in which there is only one. A commercial preparation of this acid (Schuchardt's) was used; it is not probably possible to obtain strictly pure linoleic acid and this specimen, as was expected, gave neither the theoretical molecular weight nor the theoretical iodine value. But its iodine value was more than 186, and it therefore clearly contained a large amount of acid more unsaturated than oleic or erucic and serves as the first test of the theory that if it is the unsaturated carbon atoms that confer on these acids their hemolytic properties, the more of such atoms there are in an acid the more actively it should cause hemolysis.

The result of the experiment shown in table V, which was confirmed on other occasions, would show that the minimum hemolytic dose of the sodium salt of linoleic acid, lying as it does between 0.037 and 0.025 of a milligram, is not smaller than, but approximately the same as, that of the standard sodium oleate.

Lamar (5) has also compared the hemolytic power of linoleic acid and its potassium salt with that of oleic acid and sodium and potassium oleates. For sodium oleate, as pointed out above, our figures agree very closely, but he found linoleic acid to be more active than oleic, and its potassium salt to be three or four times as active as the oleates. This discrepancy is difficult to account for. But that the hemolytic power of unsaturated fatty acids is not related to the unsaturated carbon atoms in the way that his results would probably suggest will be clear from what follows.

The next point investigated was the hemolytic properties that result when the unsaturated carbon atoms of oleic acid become saturated by the introduction of chlorine, bromine, or iodine, or of hydroxyl groups. If the pronounced difference between the lytic power of oleic acid and the corresponding saturated acid, stearic acid, is due to the chemical activity of the unsaturated carbon atoms, we should expect this difference to disappear when the oleic acid is converted into dibromostearic, chloriodostearic, monobromostearic, or dihydroxystearic.

Dibromostearic acid was prepared in the usual way by treating oleic acid dissolved in glacial acetic acid with bromine dissolved in the same solvent as long as it was absorbed, and washing the product with water till all the acetic acid and uncombined bromine had been removed.

Chloriodostearic acid was obtained by treating oleic acid with excess of Wijs's solution of iodine chloride, as in determining the iodine value.

The monobromostearic was obtained by treating oleic acid with hydrobromic acid.

Another acid with the bromine in the α position was prepared by brominating stearic acid itself; the smallest quantity of this that caused complete hemolysis under the standard conditions was almost exactly the same as in the case of the first mentioned monobromostearic acid; viz., 0.036 of a milligram, and the minimum lytic dose lies between this value and 0.018.

As is apparent from tables VI to VIII, the three saturated halogen derivatives of oleic acid are none of them appreciably less actively hemolytic than oleic acid itself; although none of them

TABLE VI.

Potassium Dibromostearate in Methyl Alcohol (1 c.c. = 4 mg.) Diluted 1 to 80 with Normal Saline (1 c.c. = 0.05 mg.).

Tube.	Lytic agent.	Result.				
		Sheep.	Rabbit.	Man.		
				A.	*B.*	*C.*
1	0.3 c.c.	Partial	Partial	Trace	Partial	Trace.
2	0.4 c.c. = 0.02 mg.	Partial	Partial	Partial	Partial	Partial.
3	0.8 c.c. = 0.04 mg.	Complete	Complete	Partial	Almost complete	Complete.
4	1.2 c.c.	Complete	Complete	Complete	Complete	Complete.

TABLE VII.

Sodium Chloriodostearate in Methyl Alcohol (1 c.c. = 4 mg.) Diluted 1 to 80 with Normal Saline (1 c.c. = 0.05 mg.).

Tube.	Lytic agent.	Result.
1	0.3 c.c.	Trace.
2	0.4 c.c. = 0.02 mg.	Almost complete.
3	0.8 c.c. = 0.04 mg.	Complete.
4	1.2 c.c.	Complete.

TABLE VIII.

Sodium Monobromostearate in Methyl Alcohol (1 c.c. = 3.27 mg.) Diluted 1 to 80 with Normal Saline (1 c.c. = 0.041 mg.).

Tube.	Lytic agent.	Result.
1	0.3 c.c.	Partial.
2	0.4 c.c. = 0.016 mg.	Partial.
3	0.8 c.c. = 0.032 mg.	Complete.
4	1.2 c.c.	Complete.

contain any unsaturated carbon atoms, the lytic dose under the standard conditions adopted for this work is practically the same as that of oleate, in the neighborhood of 0.03 of a milligram. Indeed the volume of equimolecular solutions necessary for complete hemolysis is actually smaller in the case of the salts of these acids than in the case of sodium oleate. This disposes of the idea which has been current, that oleic acid is the potent hemolytic agent that it is on account of the chemical reactivity of the unsaturated carbon atoms that alone distinguish it chemically from stearic acid. The saturation of these atoms with halogens leaves the molecule as strongly hemolytic as before.

On the other hand, the introduction of two hydroxyl groups where there are two vacant carbon bonds in oleic acid at once reduces the hemolytic power, at least as much as the two hydrogen atoms that in stearic acid have taken these positions. The product dioxystearic acid is a solid with a relatively high melting point, and it is easy to suggest that the change thus effected in the hemolytic properties of the molecule is the result of the change in its physical nature; at any rate the loss of hemolytic power is not due to the loss of the chemical properties of the unsaturated carbon atoms. The preparation of dioxystearic acid employed was made from oleic acid obtained from the pig's liver by oxidizing it in the cold with

alkaline permanganate; its preparation and properties are described by Hartley (6) to whom I am indebted for the use of his specimen.

So far the tests described above have been carried out with pure or practically pure single substances. Tables X and XI contain the results of other experiments in which the theory is tested that if unsaturated fatty acids are hemolytic because they are unsaturated, the more unsaturated they are the greater their hemolytic activity should be.

TABLE IX.

Sodium Dihydroxystearate in Methyl Alcohol (1 c.c. = 3 mg.).

Tube.	Lytic agent.	Result.
1	0.05 c.c.	Partial.
2	0.1 c.c.	Partial.
3	0.2 c.c.	Partial.
4	0.4 c.c.	Partial.
5	0.8 c.c. = 2.4 mg.	Partial.

TABLE X.

Sodium Soaps of Mixed Fatty Acids Obtained from Cod Liver Oil Dissolved in Methyl Alcohol (1 c.c. = 2.53 mg.) Diluted 1 to 80 with Normal Saline (1 c.c. = 0.03 mg.).

Tube.	Lytic agent.	Result.
1	0.3 c.c.	Partial.
2	0.4 c.c. = 0.012 mg.	Partial.
3	0.8 c.c. = 0.024 mg.	Complete.
4	1.2 c.c.	Complete.

TABLE XI.

Sodium Soaps of Mixed Fatty Acids Obtained from Linseed Oil Dissolved in Methyl Alcohol (1 c.c. = 2.78 mg.) Diluted 1 to 80 with Normal Saline (1 c.c. = 0.03 mg.).

Tube.	Lytic agent.	Result.
1	0.3 c.c.	Partial.
2	0.4 c.c. = 0.012 mg.	Partial.
3	0.8 c.c. = 0.024 mg.	Complete.
4	1.2 c.c.	Complete.

Cod liver oil is known to be composed of the glycerides of many fatty acids, some of which are much more unsaturated than oleic acid. A sample of the oil was saponified, the fatty acids separated, and their iodine value found to be 162. Had the only acid present been oleic this value would have been 90. This gives an idea of the extent to which acids more unsaturated than oleic acid were present

in the mixture. A solution of the sodium soaps was prepared and used in the strengths indicated in table X.

TABLE XII.

Minimum Completely Hemolytic Dose in Milligrams for 0.5 c.c. of a 5 Per Cent. Suspension of Red Blood Cells of the Sheep.

Sodium soaps of	Iodine value.	Mg.
Oleic acid	87.5	0.03
Palmitic acid		> 0.04
Erucic acid	61.8	0.07
Linoleic acid	186.6	0.037
Dibromostearic acid		0.04
Chloriodostearic acid		0.04
Monobromostearic acid		0.03
α bromostearic acid		0.03
Dihydroxystearic acid		2.4
Mixed acids of cod liver oil	162.0	0.024
Mixed acids of linseed oil	180.0	0.024

TABLE XIII.

Minimum Hemolytic Dose in Milligrams for 0.5 c.c. of a 5 Per Cent. Suspension of Sheep Corpuscles.

Case No.		Fraction 1 soluble in acetone.	Fatty acids from fraction 1.	Fraction 2 precipitated by acetone.	Fatty acids from fraction 2.
1. Cancer of prostate	Heart	>0.14	0.4*		
	Liver			>4.0	0.2
2. Cancer of prostate	Liver	0.04	0.022*	0.05	0.02
	Liver	0.4	0.14	0.35	0.6
3. Pneumonia	Mucosa of small intestine	0.0 0.21	0.18*		
4. Anemia not pernicious in type	Liver	>6.5	0.1	5.5	0.1
	Mucosa	0.11	0.11		
5. Pernicious anemia	Heart	0.6		2.0	
	Kidney	3.0	0.37	2.7	
	Liver	>9.0	0.3* 0.28	2.5	0.1
	Mucosa	1.7	0.3* 0.17		
	Spleen	1.3	0.45* 0.17	>3.4	0.07

Similarly linseed oil was used for the experiment to which table XI refers. The fatty acids obtained from this source had the iodine value 180, and were therefore still more largely composed of the acids more unsaturated than oleic acid than in the case of cod liver oil.

The results in tables X and XI show that the hemolytic power of these acids was somewhat stronger than that of sodium oleate, but on other occasions on which the experiments were repeated results were obtained in which the minimum hemolytic dose was larger than that shown in the tables, and no material difference therefore was demonstrable between the lytic properties of these acids and of sodium oleate, not sufficient at any rate to weaken the evidence given above for the statement that unsaturated fatty acids do not owe their activity as hemolytic agents to the chemical activity conferred on them by the unsaturated carbon atoms, whatever the true cause for their hemolytic properties may be.

We come now to the question whether toxic hemolysins of a fatty nature can be found in the body of patients dying of pernicious anemia, or in the subjects of other toxic forms of anemia.

Faust and Tallquist state that from 1 to 5 mg. of erucic, crotonic, or cinnamic acid causes intense or complete hemolysis of 10 c.c. of a 2 per cent. suspension of ox corpuscles in two hours at 37° C., and in intensity of action they are equal to oleic acid. They also state that the sodium salts of oleic and erucic acid are as strongly lytic as the free acids, while stearic and palmitic acids caused no hemolysis of red blood corpuscles of any species examined, when added up to any amount (*"beliebige Menge"*) as sodium salts. These statements are clearly not intended to be exactly quantitative. But if 10 c.c. of a 2 per cent. suspension is the same as 4 c.c. of a 5 per cent. suspension, then the dose of these substances as given by them is on the scale used in this paper, 1/8 to 5/8 mg., which is from four to twenty times too high in the case of oleic acid. A difficulty that has been generally felt in accepting the explanation given by these authors of the origin of this form of anemia is that oleic acid is constantly present in the body in considerable quantities. The blood, according to Hoppe Seyler, contains in the horse and dog up to 0.12 per cent. of soap, and in man, according to Kobert, as Meyerstein points out, the same amount. And it would seem that the protective agents existing in the serum, which Meyerstein and others have studied, cholesterol, neutral fats, and lipoids, ought to account for immunity from damage to the corpuscles even with considerable variations in the amount of oleate in the blood.

The other observers who have endeavored to ascribe the hemolysis in toxic anemias to fatty hemolysins have not confined themselves so precisely to one particular substance. Berger and Tsuchiya (7) prepared a 2 per cent. emulsion in normal saline of the ether extract of the mucous membrane of stomach and intestines from a normal human subject and also from a patient that died of pernicious anemia. In the former case five drops (of which forty-five went to 1 c.c.) were necessary to lake completely 5 c.c. of a 2 per cent. suspension of human corpuscles, or 2 c.c. of a 5 per cent. suspension: therefore 0.5 c.c. of a 5 per cent. suspension would require one fourth of this amount (1/4 of 5/45 of 20 mg.) or 0.55 mg. In the latter case, of the extract from the mucosa in

pernicious anemia one fifth of this amount was sufficient; that is, 0.11 mg. for 0.5 c.c. of 5 per cent. suspension, or about four times as much as would have been necessary had the extract consisted of sodium oleate or oleic acid. Their figures show a noticeable difference between the hemolytic properties of the normal extract and the abnormal, but even in this latter case it would not appear that the potency of the material obtained in pernicious anemia was so great as to warrant far reaching conclusions.

Joannovics and Pick prepared from the liver in acute yellow atrophy in phosphorus poisoning in man, the dog, and the rabbit, and in dogs poisoned with toluylene diamine, an extract soluble in ether, in acetone, and in methyl alcohol, which they maintain was by means of these solvents freed from simple glycerides and also from complex fatty substances containing phosphorus, and was therefore substantially composed of fatty acids. Of this material the minimum completely hemolytic dose for 0.5 c.c. of a 5 per cent. suspension of dog's red blood corpuscles was as follows:

Acute yellow atrophy	0.013 mg.
Phosphorus poisoning (man)	0.062 mg.
Phosphorus poisoning (dog)	0.112 mg.
Phosphorus poisoning (dog)	0.065 mg.
Phosphorus poisoning (rabbit)	0.105 mg.
Phosphorus poisoning (rabbit)	0.088 mg.

The fatty acids found free in the liver in these five cases of phosphorus poisoning had therefore from a half to a quarter of the hemolytic power of sodium oleate or oleic acid, whereas in the case of acute yellow atrophy it was greater than this, about twice as great. Although, therefore, the effect of phosphorus did not bring to light any evidence for the belief that in the decomposition of the unknown fatty complexes of liver cells substances might be liberated with greater lytic properties than might well belong to the simple products of the hydrolysis of common fat, nevertheless the case of yellow atrophy bears out this conception. But if this is the explanation of the hemolysis in pernicious anemia, if pernicious anemia is a sort of chronic yellow atrophy, some explanation is required to account for the fact that the extensive destruction of liver tissue in phosphorus poisoning gives such different results. Joannovics and Pick draw different conclusions from their findings, both in these cases and in the case of extracts obtained from the liver in animals poisoned with toluylene diamine. In these latter experiments the data given do not allow of an exact comparison on these lines. The volume of the methyl alcoholic solution necessary for complete hemolysis is given, but not the amount of extract contained in that volume, but the authors themselves do not suggest that these results were more striking than those obtained in the cases of phosphorus poisoning, so we should not be justified in concluding that this was the case. More recently, however, Maidorn (8) has published results of experiments of this same nature, in which the method of preparing the extracts was the same as that employed by Joannovics and Pick, and he states that in his experiments the extract soluble in acetone from 100 gm. of liver, the weight of which is given, was made up

to 100 c.c. of solution in methyl alcohol; a part, doubtless, of this extract did not dissolve in methyl alcohol, but we are not told how much did dissolve. Taking, however, the amount of extract soluble in acetone that it was necessary to use in order to get complete hemolysis in 0.5 c.c. (the author used 0.2 c.c.) of 5 per cent. sheep red blood corpuscles, we obtain the following figures:

	Extract in mg.
1. Normal liver (dog)	1.06
2. Normal liver autolyzed 48 hours (dog)	0.33
3. Acute toluylene diamine poisoning (dog)	1.60
4. Acute toluylene diamine poisoning after removal of spleen (dog)	3.80
5. Chronic toluylene diamine poisoning (dog)	1.50
6. Chronic toluylene diamine poisoning after removal of spleen (dog)	1.90
7. Acute phosphorus poisoning (dog)	2.30
8. Acute phosphorus poisoning after removal of spleen (dog)	4.80
9. Chronic phosphorus poisoning (dog)	7.40
10. Chronic phosphorus poisoning after removal of spleen (dog)	0.70

In each case we must suppose that only a certain fraction of the amounts dissolved in methyl alcohol; but for the hemolytic potency of that which did dissolve to be of the order of the hemolytic potency of oleic acid, we must suppose that this fraction was never larger than 1/10 (experiment 2) and ranged down to 1/250 (experiment 9). If as little of the acetone extract as this went into solution in methyl alcohol the author would surely have commented on the fact. It is difficult, therefore, to elicit from these results any evidence for the formation of hemolysins in these conditions, such as the author seems to have in mind; that is, so powerful as to introduce a new factor into the pathology of anemia.

In order to test the point with material obtained from the human subject in health and in pernicious anemia the following experiments were carried out.

Extracts were prepared as follows: The organ freed from all visible adipose connective tissue was minced, weighed, and treated with about 100 to 150 c.c. of alcohol for every 100 gm. of material. After twenty-four hours the aqueous alcoholic fluid was strained off on muslin, pressed out by hand, and discarded; the tissue was then shaken with the same volume of alcohol again; the alcohol pressed out in a screw-press the next day was kept, and similarly two or three further portions of alcoholic solution, obtained on the following days, were all mixed, filtered, evaporated *in vacuo* at about 40° C. to dryness, and the residue taken up in ether and filtered. After this extraction with alcohol the tissue, now practically free from water, was similarly extracted with ether, the ether similarly evaporated, and the residue taken up in ether and filtered. This ethereal solution was added to that obtained from the alcohol extraction and the mixture precipitated with acetone. The mixture of acetone and ether, filtered off and evaporated *in vacuo* at about 40° C., gave a residue which was taken up in acetone and filtered (fraction 1). The small amount which did not dissolve in

acetone, added to that which had been precipitated by acetone, was dissolved in ether and precipitated again once or twice with acetone. In this way finally a second fraction was obtained insoluble in acetone (fraction 2). These two fractions were dissolved in methyl alcohol, neutralized to phenolphthalein with N/10 soda, the amount of dissolved material in a measured quantity of each solution was determined, and then the hemolytic power of each solution ascertained. A portion also of each fraction was saponified with alcoholic potash, made acid, and the fatty acids, thus set free, were shaken out with petroleum ether, and examined as to their hemolytic power after neutralization with soda. Solutions were obtained in this way from five cases.

Case 1. A man who died after operation for cancer of the prostate; there were no metastases and no cachexia.

Case 2. Cancer of the prostate with metastases and some cachexia.

Case 3. Acute lobar pneumonia.

Case 4. Anemia not typically pernicious, color index low, no signs of regeneration, no gastro-intestinal symptoms.

Case 5. A man who died of pernicious anemia, showing the typical blood picture of the aplastic type (1.2 to 1.6 million red corpuscles with high color index and no signs of regeneration, except a few nucleated red corpuscles) in this, his third attack of illness. He exhibited intestinal symptoms which were, however, not severe.

The first four cases were thus controls and should make clear the difference between the hemolytic properties of the fatty substances obtainable from the tissues in typical pernicious anemia (case 5) and those found in other conditions, if such a difference exists and if in pernicious anemia the disease is due to hemolysis by toxic fatty substances present in the tissues.

In table XIII the results obtained with material from these cases are summarized. Where the figures given are preceded by the sign $>$, the actual amount that caused complete hemolysis was not determined; the largest amount actually used did not have this effect and that figure is given. In every instance this is sufficient to prove that there was no unusually potent agent operating.

The figures in the second column (fatty acids from fraction 1) are in some cases marked with an asterisk. Fraction 1 would contain, in addition to simple glycerides and free fatty acids, cholesterol and its esters, if present. In most instances the portion of fraction 1 that had been saponified was shaken with light petroleum two or more times in alkaline solution to remove cholesterol and unsaponified matter; in some instances the products of saponification were tested both before and after this treatment, in others only after it.

The asterisks indicate tests where the treatment had not been carried out. The protective action of cholesterol which has been studied by Meyerstein and others is illustrated where the test was made both before and after the removal of unsaponifiable substances.

The examination of the figures as a whole shows that the hemolytic action of the substances soluble in acetone and of those precipitated by it is far smaller than that of the fatty acids that can be obtained from them; and further that the action of these fatty acids is generally not as strong as would be that of pure oleic acid by itself. And more especially the figures obtained in the case of the tissues in pernicious anemia fall entirely within the limits of those obtained similarly in other conditions and fail absolutely to give any indication of the presence of hemolytic toxins of the kind which have been supposed by some to be present and to be the cause of the disease.

It is true of course that the treatment to which the material was subjected before the fatty acids were finally isolated for the carrying out of these tests can hardly have been without effect upon the chemical constitution of some of the more sensitive ones. Although all evaporations were done in flasks from which air was removed by a fine stream of carbonic acid under low pressure, and at a low temperature, still the extraction of the tissues with alcohol and with ether necessarily lasted several days, and the action of air at the room temperature during this time within stoppered vessels was not excluded; and also, of course, the saponification with alcoholic potash[1] is known to be accompanied by changes in unsaturated acids. It may therefore be that the acids as used for the tests were in part different from those originally present in the substances contained in the cells. The changes known to be brought about by such operations, partial oxidation of unsaturated acids, and consequent lowering of their iodine value, would probably, as the experiments with dihydroxystearic acid given in the earlier part of this paper show, tend to diminish the hemolytic power of the acids finally obtained.

[1] In order to avoid heating with alcoholic potash, in one experiment the saponification by means of hepatic lipase was employed for the liberation of the fatty acids. Although this did not yield results other than those given in the table, this method has not yet been adequately tried out in the experiments here recorded and is to be submitted to further tests in this laboratory.

But the oxidized acids being insoluble in petroleum ether would be removed in the final operation for obtaining the fatty acids. And we find that at any rate in the case of pernicious anemia the fatty acids obtained have a lower hemolytic action than oleic acid, excepting only those obtained from the liver, which behave about as they would if they were merely oleic acid.

CONCLUSIONS.

1. The smallest amount of the sodium soaps necessary for the complete hemolysis of 0.5 of a cubic centimeter of a 5 per cent. suspension of the red blood corpuscles of the sheep, ox, rabbit, dog, or of man, is about the same,—0.03 of a milligram in the case of the following acids: oleic, linoleic, dibromostearic, chloriodostearic, and two isomeric monobromostearic acids; in the case of erucic acid about twice as much of the soap was found to be necessary; in that of palmitic or of dihydroxystearic acid more than ten times as much.

2. The minimum hemolytic quantity of the sodium soaps of the highly unsaturated acids obtained from cod liver oil and from linseed oil is only very slightly less than that of sodium oleate.

3. It follows, therefore, from these results that hemolysis by unsaturated fatty acids is not more active in proportion to the degree to which these acids are unsaturated, nor is it diminished when the unsaturated carbon atoms are saturated by halogens. It is, on the other hand, greatly diminished when they are converted into the corresponding hydroxyl acids, which are hemolytic only to the same degree as the saturated acids.

4. The idea that toxic hemolysis, in disease, in poisoning by phosphorus or toluylene diamine, results from the liberation of specially hemolytic fatty acids from the fatty complexes of disintegrating cells is not well supported by evidence; none of the fatty acids, still less any of the fatty complexes from which these acids can be obtained in any of the organs examined, either in this work or in the work of others that has preceded it, show on analysis any evidence for the existence of fatty acids more toxic than the common oleic acid which is constantly being set free by hydrolysis from common fat in health.

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PNEUMONIC LESIONS CAUSED BY BACILLUS MEGATHERIUM.*

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INTRODUCTION.

In previous communications[1] on experimental pneumonia we showed that there are fundamental differences in the character of the pneumonic lesions produced by intrabronchial insufflation of different microörganisms. The bacteria thus far used may be divided into two groups. The lesion produced by one group resembled that of lobar pneumonia, while the lesion produced by the other group resembled that of lobular pneumonia. We shall speak only of one organism, the main representative, of each group: thus the pneumococcus produced lobar pneumonia and the streptococcus lobular pneumonia. Besides the known differences in their gross appearance, the two kinds of lesions were found to differ in two important points: (1) the exudate of experimental lobar pneumonia contained a great deal of fibrin, while that of lobular pneumonia had comparatively little; (2) in lobular pneumonia the leucocytic infiltration invaded the framework of the lungs (the walls of the alveoli and the interstitial septa), while in lobar pneumonia the framework remained free from infiltration. In a further study of the subject it was found that the insufflation of non-virulent pneumococci gave rise to a pulmonary lesion which, in gross appearance, resembled lobar pneumonia. Microscopic examination showed a further resemblance to lobar pneumonia by the absence of leucocytic infiltration of the framework. Fibrin, however, was very scanty in the exudate of the lesions caused by a non-virulent pneumococcus. It differed further from the pneumonia produced by a virulent pneumococcus in that living bacteria were absent from

* Received for publication, June 10, 1913.

[1] Wollstein, M., and Meltzer, S. J., *Jour. Exper. Med.*, 1912, xvi, 126; 1913, xvii, 353, 424.

the blood of the animals. Finally the pneumonic lesion produced by the avirulent pneumococcus generally progressed more slowly and its resolution began earlier than was the case with the lesions produced by the virulent pneumococcus.

The intrabronchial insufflation of a culture of virulent pneumococcus killed by heat produced a mild, patchy, superficial inflammation of the lung having no similarity to the lesions produced by the living pneumococcus, virulent or non-virulent.

The experiments have thus shown that while the character of the exudate varied materially with the virulence of the pneumococcus, the avirulent organism caused a lesion which has the gross appearance of a lobar pneumonia. However, an avirulent pneumococcus is still a pathogenic organism, and the question presented itself: Could unmistakable saprophytic organisms cause a pneumonic lesion, and what would be the nature of that lesion? This was the problem. We tried to solve it by introducing *Bacillus megatherium* into the lungs of dogs by the method of intrabronchial insufflation previously described. Ten experiments were made, the results of which are the subject of the present brief communication.

EXPERIMENTAL PART.

Bacillus megatherium is a Gram-positive, spore-bearing, motile bacillus which ferments glucose, lactose, saccharose, mannite, and maltose, without forming gas. We found that when injected intravenously into rabbits and intraperitoneally into guinea pigs and rabbits even large doses (one cubic centimeter of broth cultures twenty-four hours old) caused no discomfort to the animal. Guinea pigs killed twenty-four hours after an intraperitoneal inoculation of a whole agar slant one day old showed a very small amount (less than 0.2 of a cubic centimeter) of turbid peritoneal exudate. In smears made from this fluid there were many polymorphonuclear leucocytes but no bacilli. Fluid removed from the animal's abdomen within half an hour after the injection showed many bacilli and few leucocytes. Two and three hours after the inoculation there were more leucocytes and some phagocytosis. Cultures from the earlier specimens grew profusely but from the fluid obtained after twenty-four hours the growth was sparse.

Subcutaneous injections of large doses did not cause abscess formation. After twenty-four hours there was congestion and edema at the site of inoculation. In smears some polymorphonuclear leucocytes and many bacilli were seen, the cultures growing well.

The bacillus has been found in the intestinal tract of infants,[2] having been taken with food, and causing no ill effects. The only attempt to make *Bacillus megatherium* pathogenic for animals has been made by Vincent,[3] who inserted collodion sacs containing cultures of the bacillus into the abdominal cavity of guinea pigs, and he states that after the fourth passage it had become pathogenic for these animals.

The broth cultures of *Bacillus megatherium* which were introduced into the lungs of the dogs were twenty-four hours old and practically free from spores. Doses of fifteen to twenty-five cubic centimeters were given. None of the animals died, nor did they seem to be ill. Four dogs were killed in twenty-four hours after inoculation, two after forty-eight hours, two after three days, one after four days, and one after five days.

Within twenty-four hours after inoculation the lungs presented a lesion which was always limited to one lobe; in two cases it was the right posterior, and in the other two the left posterior. The greater part of this lobe was dark red, heavy, firm, but not swollen. There was no exudate on its pleural surface. On section blood-stained fluid exuded and the cut surface was found to be red, evenly consolidated, and very moist.

Microscopically the lesion was one of intense leucocytic exudation. Polymorphonuclear leucocytes, many with fragmenting nuclei, filled the alveoli and the small bronchi, whose walls, however, remained free from infiltration. The blood vessels were distended with blood cells, both in the alveolar walls and in the interlobular septa. At the periphery of the lesion some alveoli contained serum only. No fibrin was seen. The connective tissue framework was not infiltrated.

No bacilli were found free in smears made from the solid lung, nor was there any evidence of phagocytosis. In cultures no growth

[2] Wollstein, M., *Am. Jour. Dis. Child.*, 1912, iv, 279.

[3] Vincent, H., *Ann. de l'Inst. Pasteur*, 1898, xii, 785.

was obtained from the heart's blood in any case of the entire series, nor from the lungs in nine of the ten cases. In the tenth case two colonies of *Bacillus megatherium* developed from the consolidated left posterior lobe of an animal killed twenty-four hours after inoculation of a dose of fifteen cubic centimeters. The pneumonia in this series of experiments was strictly a local lesion. The animals had shown scarcely any rise of temperature; only in two cases did the rise reach 1° C.

Two days after insufflation of fifteen to twenty-five cubic centimeters of a broth culture of *Bacillus megatherium* the pulmonary lesion was no more extensive or intense than it had been in twenty-four hours. In the case of the smaller dose (fifteen cubic centimeters) resolution had already begun.

On the third day resolution had progressed so far that only small solid areas remained, and on the fourth day there was congestion but no consolidation.

DISCUSSION AND SUMMARY.

The essential points of our results are as follows: The saprophytic *Bacillus megatherium* produced a definite pulmonary lesion which in gross appearance resembled the lesion of lobar pneumonia. The lesion was one of intense leucocytic exudation which, as in some other cases of experimental lobar pneumonia, did not invade the framework of the lung. But the exudate contained no fibrin. There were practically no bacilli, either in the heart's blood or in the lung. The growth of two colonies from one of the cases hardly changes the rule. The lesion did not progress after twenty-four hours; nor did it show a definite increase in extent or intensity with the increase in quantity of the injected cultures. The resolution of the exudate began practically at the end of twenty-four hours and was far advanced on the third day. Although there was considerable leucocytic infiltration there was no phagocytosis apparent twenty-four hours after inoculation.

Taking all the facts into consideration one gains the impression that the lesion produced by the saprophytic *Bacillus megatherium* differs only quantitatively from that produced by an avirulent pneumococ-

cus. The fact stands out prominently that a saprophytic bacterium is capable of producing a pneumonic lesion similar in gross appearance to that of mild lobar pneumonia. It is not improbable that other saprophytes may be capable of producing pneumonic lesions which, in specific instances, may resemble lobular pneumonia.

THE CHARACTER OF THE PNEUMONIC LESIONS PRODUCED BY INTRABRONCHIAL INSUFFLATION OF VIRULENT STREPTOCOCCI.*

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INTRODUCTION.

In our first studies[1] on the effect of intrabronchial insufflation of the streptococcus in dogs, we found that the nature of the pneumonic lesion produced by this organism was one which in human pathology is usually described as lobular pneumonia. In the insufflation experiments of Lamar and Meltzer[2] with the pneumococcus, the lesion produced was that of a lobar pneumonia. The conclusion which presented itself was that the different organisms cause experimentally different pneumonic lesions. In a later communication we[3] enumerated in detail the differences in the two forms of pneumonic lesions resulting experimentally from the method of intrabronchial insufflation. The question arose whether the differences in the lesions were indeed specific; that is, whether the pneumococcus always produces a lobar pneumonia. The two series of experiments did not exclude the possibility that the difference in the results was merely due to a difference in the degree of virulence of the two species of organisms employed in the two investigations. The pneumococcus employed in the experiments of Lamar and Meltzer was highly virulent. It was fatal to mice in doses of 0.000,001 of a cubic centimeter. The strain of streptococcus which we employed in our first experiments and which had been isolated from a fatal case of bronchopneumonia caused the death of a mouse weighing fifteen grams only after the intraperitoneal injection of

* Received for publication, June 28, 1913.

[1] Wollstein, M., and Meltzer, S. J., *Jour. Exper. Med.*, 1912, xvi, 126.

[2] Lamar, R. V., and Meltzer, S. J., *idem*, 1912, xv, 133.

[3] Wollstein, M., and Meltzer, S. J., *idem*, 1913, xvii, 353.

0.5 of a cubic centimeter. We[4] tried to answer the question in part, at least, by studying the character of the pneumonic lesions which followed insufflation of a non-virulent pneumococcus isolated from the blood of a patient with lobar pneumonia who recovered. In the present series of experiments we attempted to meet the same question by studying the character of the pneumonic lesion produced by a strain of streptococcus whose virulence is comparable with that of the strain of the pneumococcus employed in the experiments of Lamar and Meltzer, inasmuch as it is also fatal to mice in doses of 0.000,001 of a cubic centimeter.

EXPERIMENTAL DATA.

From a fatal case of purulent leptomeningitis a streptococcus was isolated from the spinal fluid and from the heart's blood at autopsy. The coccus grew in chains of six to twenty elements, did not ferment inulin, and was not dissolved in rabbit bile. It formed the streptococcus type of capsules. On blood agar plates a distinct hemolytic zone surrounded the small round colonies. From the first generation on agar broth tubes were inoculated, and after twenty-four hours' growth 0.01 of a cubic centimeter was injected intraperitoneally into a white mouse weighing fifteen grams. The following morning the mouse was dead, and the streptococcus was recovered from the heart's blood in pure culture. Smaller doses were inoculated into successive mice, until 0.000,001 of a cubic centimeter killed the animals in from twenty-four to thirty-six hours. Broth cultures from this virulent streptococcus were used in the following series of experiments on dogs. From time to time the coccus was reinoculated into mice in order to test its virulence, which was thus kept undiminished throughout the period of our experiments.

Seventeen dogs were inoculated by intrabronchial insufflation of broth cultures eighteen to twenty-four hours old, in doses of fifteen to twenty-five cubic centimeters. No one of the animals died, nor were they very ill. The temperature generally rose within six or seven hours after the injection, but in almost every case it had fallen quite or almost to normal by the following morning. The highest rise recorded was 40.8° C.

[4] Wollstein, M., and Meltzer, S. J., *loc. cit.*, 1913, xvii, 353.

Four dogs to which fifteen cubic centimeters had been given were killed in twenty-four hours. In all the lesion was confined to the right lower lobe, the greater part of which was somewhat swollen, heavy, mottled with dark and paler areas, while on section the moist cut surface was distinctly that of a lobular pneumonia in which aerated, pink areas of small extent were present among the more solid peribronchial, dark red masses of smaller and larger size. There was no pleural exudate present in any of the cases. Smears from the lung showed many cocci in very short chains, almost all lying outside of the leucocytes. There was practically no phagocytosis. Cultures from the lung and from the heart's blood gave pure growths of streptococci. The bronchial lymph nodes were swollen, moist, and soft.

Three dogs received twenty cubic centimeters of the culture and showed a more extensive lesion in twenty-four hours. In one of these animals the subcardiac lobe was involved as well as the right lower, and in the others the entire lobe was solid, the picture being that of a pseudo-lobar bronchopneumonia. Only one case showed a narrow strip of fibrinous exudate on the pleura, along the posterior border of the subcardiac lobe. In two of these cases the consolidated lung contained areas that were as firmly solidified as were the control specimens of pneumococcus pneumonia, but these solid areas were less extensive and were separated, or interrupted, by softer, lighter areas of aerated, congested, or edematous lung substance.

In two dogs a blood culture from the jugular vein was taken every half hour for two hours after the insufflation of twenty cubic centimeters of streptococcus culture. In one of the animals a growth appeared in the culture made after one hour, while in the other no bacteriemia was apparent two hours after the cocci had been injected into the bronchus. Both dogs, however, showed streptococci in the blood just before death twenty-four hours after inoculation.

Three dogs were killed two days after a dose of fifteen cubic centimeters of streptococci had been given them. In two the right lower lobe showed a bronchopneumonia in its posterior third, while in the other dog the subcardiac lobe was also involved. The lesions were not more intense than were those studied at the end of twenty-

four hours. There was no pleurisy present. Smears from the lungs showed few cocci; the cultures from the heart's blood and lungs were less profuse than they had been in dogs killed on the first day after inoculation.

In one case a dog to which fifteen cubic centimeters had been given was allowed to live until the sixth day, when a large area of resolving bronchopneumonia was found in the right lower lobe, and a growth of streptococci was obtained from it. The heart's blood gave no growth.

Four dogs received twenty-five cubic centimeters and were allowed to live seven days. In two cases a resolving area of bronchopneumonia was found in one of the lower lobes. In the other two dogs the right lower lobe was merely darker red in color than the rest of the lungs. No growth took place from the lungs or heart's blood.

Two dogs were kept alive thirteen days after intrabronchial insufflation of fifteen cubic centimeters of a streptococcus broth culture. Their lungs were well aerated, elastic, and pink in color, showing no evidence of any pneumonic lesion.

Microscopic Examination.—Microscopic examination of the pulmonary lesion found twenty-four hours after the intrabronchial inoculation of fifteen cubic centimeters of a broth culture of streptococcus into a dog showed that the alveoli were filled with an exudate composed very largely of polymorphonuclear leucocytes, many swollen epithelial cells and few red blood cells. Fibrin was present in very small amounts in the alveoli, but about the larger blood vessels there was often an appreciable amount of it. Toward the periphery of the lesion it sometimes happened that an area of intense congestion with intra-alveolar hemorrhage and edema intervened between the consolidated lung and the aerated portions. In such areas fibrin was not uncommon, due to the coagulation of the exuded blood. Within the alveoli of the pneumonic lesion proper, however, the amount of fibrin was never as large as is the case in lesions caused by virulent pneumococci in the dog's lung. The capillaries as well as the larger blood vessels were very much congested. The alveolar walls as well as all the connective tissue septa were densely infiltrated with polymorphonuclear leucocytes. The

small bronchi showed much swelling and peeling of their epithelial lining cells and infiltration of their walls, some of them being filled with masses of pus cells.

Streptococci in large numbers were present in the alveoli and in the bronchi, lying in heaps between the cells. Only an occasional leucocyte could be seen with cocci in its cytoplasm.

At the end of forty-eight hours the exudate was more distinctly purulent in character, and the diffuse leucocytic infiltration more marked. There was less, not more fibrin, and less edema. In some sections the alveoli were so distended with leucocytes that the capillaries in the walls were compressed, instead of distended with blood, as was the case in the earlier lesions. Streptococci were present in the alveoli, and there was some phagocytosis.

In the resolving lesions of the sixth and seventh days there was the usual evidence of macrophage activity.

DISCUSSION AND SUMMARY.

Comparing the pneumonic lesions produced by this very virulent streptococcus with those caused by a mildly virulent strain of the same organism, we find that with one exception the differences between them are only of a quantitative nature and not striking. There are the same intense leucocytic exudation into the alveoli and the leucocytic infiltration of the framework of the lungs. Macroscopically the pneumonia produced by the virulent organism was, as a rule, lobular in character. Occasionally, however, especially when large doses of the culture had been given, the gross appearance of the lesion gave the impression of a lobar pneumonia. But even in these cases the cut surface showed that the solid areas were separated by softer and lighter areas of aerated, congested, or edematous lung substance. Pleurisy was practically absent from the lungs in this series of experiments; only one case showed a narrow strip of fibrinous exudate on the pleura. Although in some instances large doses of the cultures were given and some dogs were permitted to live six or seven days, there was no mortality among the dogs in these experiments, just as there had been none among the animals experimented upon with the streptococcus of very low virulence. The course of the pneumonia caused by the virulent organism

seemed to be somewhat longer than that produced by the less virulent one; areas of resolving pneumonia were found to persist on the sixth and seventh days after the injection, and in one such instance the solid focus contained viable organisms. There was some difference in the amount of fibrin present in the exudate caused by the two strains of streptococci. Although even in these experiments the amount of fibrin present was less than that found in the lesion produced by the virulent pneumococcus, it was perceptibly larger than the very small amount found in the lesions caused by the less virulent strain of streptococcus.

It should be mentioned that there was no difference in the degree of phagocytosis observed in these two series of experiments. It was not marked in either case.

One point of difference in the two series was noted: during the first forty-eight hours after insufflation of the virulent strain of streptococcus the blood of the animals obtained from the jugular vein during life and from the heart after death contained living organisms, while in the experiments with the slightly virulent streptococcus no living cocci were recovered from the blood.

The virulence of the strain of streptococcus employed in the present investigation was, as has been pointed out, similar to that of the pneumococcus employed in the investigation of Lamar and Meltzer and in many of our own experiments, as far as mice were concerned, since both killed mice weighing fifteen grams in doses of 0.000,001 of a cubic centimeter in twenty-four to thirty-six hours. The findings in the lesions caused by these two organisms are comparable and therefore some deductions may be drawn regarding the similarity or dissimilarity of their actions. One of the first things we wish to bring out is the correction of our former statement regarding the presence of the cocci in the blood. As our new investigation shows, there is evidently no difference in this respect between the streptococcus and the pneumococcus. When both organisms are highly virulent they can be found in the circulating blood of the living dog or in the heart's blood of the dead dog during the first twenty-four or forty-eight hours after an intrabronchial insufflation.

On the other hand, the present investigation rather confirms in a

general way our first contention that the pneumonic lesions produced by the two different organisms differ distinctly in many ways. There is the difference in the mortality; even with fairly large quantities of the culture of the virulent streptococcus the mortality was nil in this series, although some of the dogs were kept alive for six and seven days. The mortality of the pneumococcus infection depends upon the quantity insufflated and may amount, as Lamar and Meltzer pointed out, to 16 per cent.

The gross appearance of the lesion produced by the pneumococcus is, as frequently stated, that of a lobar pneumonia, frequently accompanied by a fibrinous exudate on the pleura. The gross appearance of the lesion produced even by a virulent streptococcus is, as a rule, that of a lobular pneumonia and is practically never accompanied by pleurisy. In the few instances in which large quantities of the streptococcus were given and the outward appearance of the lungs approached that of a lobar pneumonia, it was found that on section of the consolidated lung the solid foci proved to be separated by lighter areas of aerated, congested lung.

In the lesions produced by the virulent streptococci the walls of the finer bronchi and the framework of the lung were markedly infiltrated with leucocytes, while in the lobar pneumonia produced by the pneumococcus the framework remained free from such infiltration.

Finally there is the unmistakable difference in the amount of fibrin present in the alveolar exudate. While in the exudate of the lesion produced by the virulent streptococcus the amount was perceptibly larger than the insignificant amount present in the lesion caused by a slightly virulent strain, it is not to be compared with the large amount of fibrin which exists in the exudate of pneumococcus pneumonia.

The several investigations which we have carried out seem to show conclusively that in general the streptococcus causes a lobular pneumonia which, besides the leucocytic intra-alveolar exudation, is characterized by a leucocytic infiltration of the lung framework, and that the pneumococcus causes a lobar pneumonia, which is practically free from leucocytic infiltration of the interstitial tissue of the lung. Furthermore, a virulent pneumococcus causes a lesion in which

fibrin is a prominent element in the exudate and that element distinguishes the exudate sharply from the exudate of the lesion caused by a virulent streptococcus in which fibrin is present only in moderate amount. It distinguishes it in a still more striking manner from the exudates of the lesions caused by non-virulent pneumococci or streptococci, in which fibrin is present only in very small amounts.

It seems that the formation of fibrin is connected in some specific way with the pneumococcus on the one hand, and with the virulence of the organism on the other. For even with the relatively small amounts of fibrin present in the exudates of lesions caused by the streptococcus there is a perceptible difference in the quantity according to the virulence of the organism.

Whether fibrin is a means which enhances virulence, or whether it is a reaction product against it, our experiments so far do not entitle us to discuss.

DISTURBANCES OF THE HEART-BEAT IN THE DOG CAUSED BY SERUM ANAPHYLAXIS.*

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Plates 42 to 45.

INTRODUCTION.

The present investigation is a continuation of the electrocardiographic study of the cardiac disturbances in the rabbit caused by serum anaphylaxis.[1] The literature on the effect of anaphylaxis on the heart of the dog is not extensive, and is entirely opposed to the idea that the dog's heart is primarily affected by anaphylaxis. Biedl and Kraus[2] exclude, on theoretic grounds, any cardiac effect during the anaphylactic reaction in dogs. Eisenbrey and Pearce[3] examined the hearts of anaphylactic dogs with a myocardiograph, but failed to detect a primary effect on the heart muscle. The ordinary tracings with the myocardiograph, however, are too condensed to permit the detection of partial heart-block, and the finer degrees of an abnormal contraction are not revealed.

The electrocardiographic method proved so satisfactory when working with rabbits, exposing cardiac disturbances which could not be recorded by the ordinary graphic method, that it seemed advisable to apply this method to the study of the cardiac activity of the dog in anaphylaxis. Findings in another series of experiments on dogs, performed by one of us, indicated that cardiac disturbance would probably be revealed by electrocardiograms, for a

* A preliminary note appeared in the *Zentralblatt für Physiologie*, 1913, xxvii, 383. Received for publication, June 26, 1913.

[1] A preliminary note was published in the *Zentralblatt für Physiologie*, 1913, xxvii, 1; a full report in the *Journal of Experimental Medicine*, 1913, xviii, 450.

[2] Biedl, A., and Kraus, R., in Kraus, R., and Levaditi, C., Handbuch der Technik und Methodik der Immunitätsforschung, Ergänzungsband, Jena, 1911, 258.

[3] Eisenbrey, A. B., and Pearce, R. M., *Jour. Pharmacol. and Exper. Therap.*, 1912, iv, 27.

certain number of dogs died acutely within twenty to forty minutes after the injection of the second dose of horse serum, and immediate autopsy showed that the heart had largely lost its irritability and contractility when stimulated mechanically or electrically. As these changes were similar to those observed in the rabbit,[4] and as the latter showed undoubted disturbances in the heart's activity when observed directly[5] or by the electrocardiograph, it seemed reasonable to expect some cardiac disturbances also in the dog.

Most investigators, in speaking of the anaphylactic reaction, use the term anaphylactic shock. The term shock, though by no means a well defined physiological entity, means either a low blood pressure (vascular shock), or a rapid feeble pulse (cardiac shock), or a combination of the two.[6] The term shock, therefore, may be applied to the anaphylactic reaction of the dog and the rabbit, but not to that of the guinea pig, for this animal dies of an asphyxia.[7]

EXPERIMENTAL PART.

The dogs used varied in weight from 6 to 14 kilos. Sensitization was produced by the subcutaneous injection of 10 c.c. of horse serum, 5 c.c. in each flank. The period of incubation lasted from twenty-two to sixty-two days. The degree of sensitization obtained varied considerably, as far as cardiac effects are concerned, though all showed the characteristic drop in blood pressure.[8] No definite relation could be detected between the length of sensitization and the toxic effect of the reinjection; outspoken cardiac effects, for example, were obtained in one set after 61, 35, 37, and 40 days, while other dogs after practically the same intervals showed no disturbances of the heart-beat.

The experiments were performed as follows: After etherization, narcosis was kept up by means of intratracheal insufflation; cannulas were then introduced into a carotid artery and an external jugular vein. The carotid blood pressure was read by means of a mercury manometer, the connecting tubing being filled with a half saturated solution of sodium sulphate. The toxic dose of horse serum, usually 20 c.c., was always injected into a jugular vein. In eight experiments this injection was repeated after the animal had partly recovered from the effects of the first one. It may be added that the horse serum employed was about nine months old. The injections required about one minute. In four experiments the vagi were cut in the neck before the second injection of horse serum was given.

The cardiac activity was recorded by means of an electrocardiograph, the

[4] Auer, J., *Jour. Exper. Med.*, 1911, xiv, 494.

[5] Auer, J., *loc. cit.*, p. 483.

[6] Howell, W. H., Contributions to Medical Research, Dedicated to Victor Clarence Vaughan, Ann Arbor, 1903, 51.

[7] Auer, J., and Lewis, P. A., *Jour. Exper. Med.*, 1910, xii, 172.

[8] The blood pressure was not recorded in the first two experiments. Both of the dogs recovered completely and were used again after some weeks.

Edelmann large model being used. The string of the galvanometer was always so adjusted that the passage of one millivolt gave a movement of 1 cm. on the curves. The electrodes were always applied to the right fore leg and left hind leg. In most experiments, non-polarizable electrodes, attached to the animal by gauze soaked in salt solution, were used. In others gold plated needles, brazed on curved, lacquered brass plates provided with a binding post, were inserted under the skin; the plates from which the wires led to the galvanometer were held in position by tapes. Both methods gave identical results. Control curves were usually made before and after the operation and, in the early experiments, also before and after the manometer recorded the blood pressure. Electrocardiograms were then taken before and after the injection and at frequent intervals subsequently, usually every fifteen seconds for several minutes,—then at less frequent intervals until all apparent effects had disappeared. The movements of the string were always carefully watched and curves taken whenever any change was noted. The time consumed by one experiment varied between ten minutes and two hours. Only one animal, No. 12, succumbed acutely during the experiment; dogs 8, 9, 10, and 16 survived for one or two days and then died. The other dogs were usually killed at the end of the experiment by injecting strophanthin intravenously. A specimen protocol is given below. All experiments were carried out in this manner.

PROTOCOL OF EXPERIMENT 8.

February 15, 1913. Tan and white dog, male; weight, 5,750 gm. The dog was etherized, a catheter introduced into the trachea, and etherization by intratracheal insufflation was begun. Electrodes were applied to the right fore leg and the left hind leg.

Time.	No. of curve.	Blood pressure.	Rate.	P–R time.	Form of complexes.
10.34 A.M.	100.1	—	167	0.10	P well formed, R of 15 mm., definite S, no T wave.
10.44 A.M.	Cannulas in external jugular and in carotid artery.				
10.45 A.M.	100.2	—	212	0.09	R of 14 mm., S deeper, split T wave.
10.46 A.M. (figure 1)	100.3	100	212	0.09	No change.
10.47 A.M.	Injection of 20 c.c. of horse serum into jugular vein begun.				
	100.4	—	207	0.10	No change.
10.48 A.M.	Injection of horse serum finished.				
	100.5	—	204	0.10	No change.
10.48¼ A.M.	100.6	25	178	0.14	P wave smaller, T less definite. Respiration regular.
10.48½ A.M.	101.1	15	167	0.16	No change.
10.49 A.M.	101.2	15	170	0.18	S wave deeper.
10.49¼ A.M. (figure 2)	101.3	20	161	0.17	No change.
10.49½ A.M.	101.4	—	163	0.16–0.17	No change. Animal quiet.
10.50½ A.M.	102.1	20	165	0.16	R of 8 mm., S 4 mm. deep.
10.51 A.M.	102.2	—	161	0.15	No change.
10.51½ A.M.	102.3	15	161	0.14–0.15	No change.
10.52½ A.M.	102.4	15	157	0.13–0.14	R of 6 mm. and broken in ascending limb. Respiration regular, dog quiet.

Time.	No. of curve.	Blood pressure.	Rate.	P–R time.	Form of complexes.
10.53¼ A.M.	103.1	10–15	158	0.12–0.13	No change. Moderately active expiration.
10.54½ A.M.	103.2	10–15	160	0.11–0.12	R of 5 mm., break in ascending limb.
10.55¼ A.M.	103.3	10	161	0.11	No change. No feces passed.
10.56¼ A.M.	103.4	10	160	0.10	No change. Respiration slower, regular.
10.57½ A.M. (figure 3)	103.5	10	160	0.09	Respiration slower.
10.59¾ A.M.	104.1	—	153	0.09	R 7 mm. high, S 3 mm. deep.
11.00 A.M.	104.2	15	154	0.09	No change.
11.01¼ A.M.	104.3	15	157	0.09	Double peak of T, less distinct.
11.02¾ A.M.	104.4	18	156	0.09	No change.
11.04 A.M.	104.5	18	158	0.085	No change.
11.07 A.M.	105.1	25	168	0.09	R wave of 8–10 mm. Dog quiet.
11.08¼ A.M.	105.2	30	165	0.09	No change.
11.10¾ A.M.	105.3	30–32	174	—	No change.
11.13¾ A.M.	105.4	32	174	0.09	No change. Dog on ½ ether.
11.16 A.M.	105.5	35	180	0.09	No change.
11.19 A.M.	106.1	40	180	0.09	No change. Dog quiet.
11.22½ A.M.	106.2	40	180		No change.
11.28 A.M.	106.3	40–45	180	0.09	No change.
11.37 A.M.		50			
11.40½ A.M.	106.4	45–50	188	0.09	No change.
11.48¼ A.M.	106.5	45–50	190	0.09	No change.
12.04 P.M.	107.1	55–60	188	0.09	No change.
12.05½ P.M.	107.2	55	185	0.09	R of 10–12 mm.
12.06 P.M.	Injection of 20 c.c. of horse serum begun.				
	107.3	—	180	0.09	R of 13 mm.
12.07 P.M.	Injection finished.				
	107.4	—	177	0.09	No change.
12.07¼ P.M.	107.5	55–60	—	—	
12.08 P.M.	107.6	55–60	182	0.085–0.09	No change.
12.13 P.M.	108.1	55–60	185	0.09	No change.
12.14 P.M.	108.2	55–60	180	0.09	No change.
12.24½ P.M.		55			Ether stopped. No change.
12.25 P.M.	108.3	55	187	0.09	No change.
12.30 P.M.	108.4	45–55	186	0.08–0.09	No change.

The wound was dressed after removal of the cannulas and after treating the wound with tincture of iodin. The dog was removed to the kennel, but was found dead on February 17, two days after the experiment.

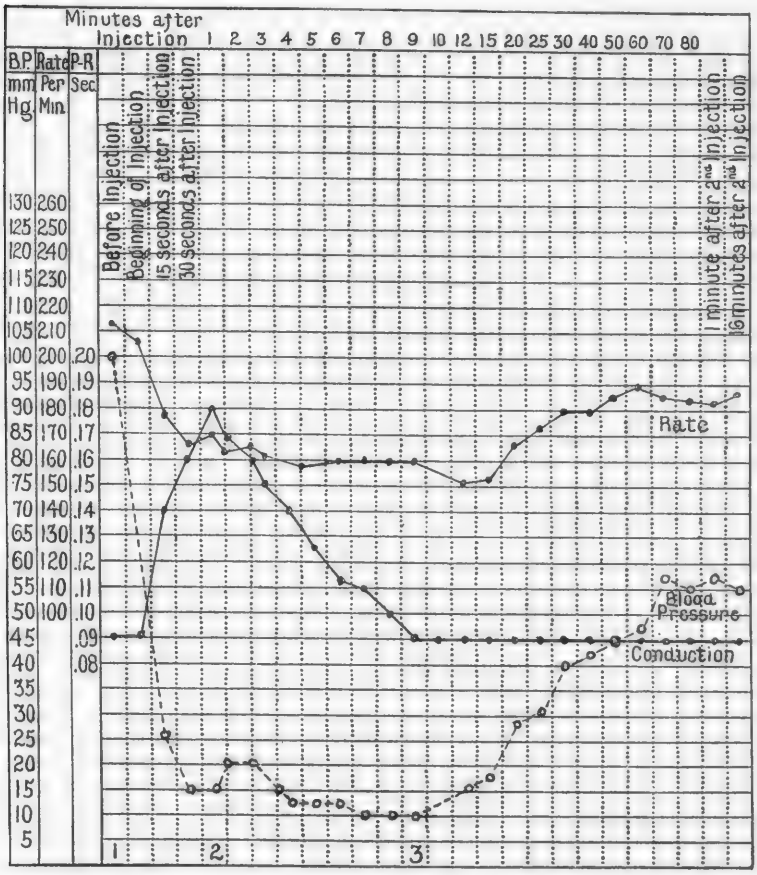
The most striking effect of the anaphylactic reaction was the well known, marked fall in blood pressure, first described by Biedl and Kraus.[9] This occurred immediately after injection in the ten experiments in which the blood pressure was observed. The average fall was 106 millimeters of mercury. The pressures before the injections averaged 126 millimeters of mercury, and the lowest

[9] Biedl, A., and Kraus, R., *Wien. klin. Wchnschr.*, 1909, xxii, 365; Arthus, M., *Arch. internat. de physiol.*, 1910, ix, 179.

pressures observed after the injections averaged 19.5. The time required for this fall of blood pressure averaged about three and one half minutes, but varied in the different experiments from one half to seven minutes. Six of the experiments showed practically no changes in the heart-beat, except changes in rate. There was no evidence of disturbance of conduction from auricles to ventricles, and the electrocardiograms showed no abnormalities in form. In six other experiments, however, definite alterations were observed in the electrocardiograms, in addition to changes in rate, which were evidently part of the anaphylactic reaction.

The dogs, in which a change of rate was the only disturbance of cardiac activity, showed almost constantly a gradual reduction of the cardiac rate throughout the experiment, and in only one instance was the rate higher after the injection than before. Thus in experiment 7, during a drop in the blood pressure from 140 to 30 millimeters of mercury, the heart rate gradually fell from 210 to 140 beats per minute, and continued at this rate until the end of the experiment, although the blood pressure rose later to 85 millimeters of mercury. In three other experiments, in one of which the vagi were cut, similar results were observed, while in the two remaining experiments, changes in rate were very slight. The six experiments with definite cardiac disturbances showed a less constant change in rate. In two a gradual fall in rate took place, while in two there was but little change. In one of the remaining two experiments (No. 8, text-figure 1) the rate fell somewhat, but rose again with the rise of blood pressure, while in the other the rate fluctuated considerably in spite of a sudden fall and continued relatively low blood pressure. In this experiment the rate rose from 175 beats per minute before the injection to 260 beats thirty seconds after the injection. In three minutes more it fell again to 150, but rose again to 212 beats three minutes later. This was the only experiment in which a sharp rise in cardiac rate accompanied the marked fall in blood pressure, but even here the increased rate was of short duration and seems to have been independent of the fall in blood pressure. It may, therefore, be stated, in general, that the changes in cardiac rate were not marked, and usually consisted of a gradual decrease which seemed independent of changes in blood pressure.

Definite disturbances in cardiac activity were seen in six experiments. These disturbances were indicated in the electrocardiograms by abnormal relations in waves of auricular and ventricular activity



TEXT-FIG. 1. Curve constructed from the records of experiment 8. The records in figures 1, 2, and 3 were obtained at the time in the experiment marked by 1, 2, and 3 at the bottom of the curve. The relation of changes in rate, blood pressure, and conduction, and the time of injection of the horse serum is shown. Upper solid line = cardiac rate per minute. Lower solid line = conduction (P-R) time in seconds. Broken line = blood pressure in mm. of mercury.

and by significant changes in the form of the ventricular portions. In five experiments there was a delay in the conduction of impulses from auricles to ventricles which resulted in the prolongation of the P-R time to more than 0.12 of a second. In two of these experiments the disturbance of conduction was so marked that partial auriculoventricular block occurred. Definite changes in form occurred in the ventricular portions of the electrocardiograms of three experiments. These changes and the disturbances in conduction occurred both in dogs with intact vagi and in others where they had been cut.

A temporary lengthening of the conduction (P-R) time occurred in two experiments (Nos. 8 and 15). In experiment 8 the vagi were intact. Before the injection the blood pressure was 100 millimeters of mercury, the rate 212 per minute, and the conduction (P-R) time 0.09 of a second. The form of the electrocardiogram was that of a normal sequential beat (figure 1). One minute after the injection of twenty cubic centimeters of horse serum, the blood pressure had fallen to 15 millimeters of mercury, the conduction (P-R) time was lengthened to 0.18 of a second, and the rate was slowed to 175 beats per minute (figure 2). During the next fourteen minutes the blood pressure and cardiac rate showed no definite variation, but the conduction time gradually shortened and had returned five minutes before any rise of blood pressure occurred to 0.09 of a second (figure 3). The conduction time showed no further change during the two hours in which curves were made. The relation of the changes in rate, in conduction, and in blood pressure is seen in text-figure 1.

In experiment 15 both vagi were cut. The injection of twenty cubic centimeters of horse serum was followed here also by a definite prolongation of the conduction time from 0.10 of a second (figure 4) to 0.14 of a second (figure 5), which continued for thirteen minutes and then returned to normal. The blood pressure fell from 115 to 32 millimeters of mercury and did not rise beyond 40 millimeters of mercury for thirty-six minutes. There was almost no change in rate throughout the experiment. The most striking feature in this experiment, however, is the gradual change in the form of the ventricular complexes. This change began about one

minute after the injection. The R waves became smaller, the S waves became more prominent, and the T waves, which were inverted or negative at first, disappeared and then increased gradually in size. The change was most marked twelve and three quarter minutes after the injection (figures 4 and 5), when the complexes began to revert gradually to the normal, so that twenty-seven minutes after the injection the electrocardiograms resembled those obtained before the injection. Experiment 16, in which the vagi were intact, showed changes similar to those seen in experiment 15, where the vagi were cut (figures 4 and 5). The change in the ventricular complexes began three and three quarter minutes after the injection and was most marked thirteen minutes later. Twenty-two minutes after the injection the ventricular complexes resembled those obtained before the injection.

In two experiments, Nos. 1 and 12, partial auriculoventricular block occurred. In experiment 1 the vagi were intact. Changes in the ventricular complexes occurred, similar to those seen in experiments 15 and 16. Before the injection the electrocardiograms were normal (figure 6). After the injection of serum the conduction (P-R) time gradually lengthened. In half a minute the R waves became smaller, and a splitting and deepening of the S waves and an increase in the size of the T waves set in. In three minutes partial block occurred, at first every eighth auricular beat failing to stimulate a ventricular contraction (figure 7). The conduction time after a blocked auricular beat was 0.12 of a second at first, but it lengthened to 0.14 of a second five minutes later, when every fifth or fourth auricular beat was blocked (figure 8). The partial block continued until twenty-two minutes after the injection, when all auricular contractions were followed by ventricular contractions, the conduction time being 0.15 of a second. The conduction time gradually improved, and, at twenty-nine minutes after the injection, was 0.12 of a second (figure 9), and the ventricular complexes again resembled those seen before the injection. In experiment 12, both vagi were cut. Before the injection the blood pressure was 145 millimeters of mercury, the rate was 217, the conduction (P-R) time was 0.08 of a second, and the ventricular complexes were normal (figure 10). Thirty seconds afterward, partial auriculo-

ventricular block appeared, when the blood pressure had fallen to 20 millimeters of mercury. Fifteen seconds later every fifth auricular beat was blocked (figure 11). The conduction (P-R) time in the cardiac cycles following the blocked auricular beats was 0.15 of a second. In the succeeding cycles it gradually lengthened until it was 0.27 of a second; then an auricular impulse was again blocked. In spite of the fact that the blood pressure remained low (10 to 18 millimeters of mercury), the sequential beat returned after two minutes. The conduction time at first was 0.20 of a second (figure 12), but five minutes later it was 0.11.

The electrocardiograms obtained in experiment 9 showed many unusual changes after the serum injection. Before the injection the blood pressure was 135 millimeters of mercury, and the electrocardiograms were normal (rate 210). One minute after the injection the blood pressure was 40 millimeters of mercury and the rate was 140. There was a definite lengthening in conduction from 0.09 to 0.18 of a second, lasting only one minute, and then a gradual diminution occurred. In six and one half minutes after the injection the P-R time was normal again and the cardiac rate was unchanged. Meanwhile, the blood pressure had fallen further from 40 to 20 millimeters of mercury. The record obtained at eleven and one quarter minutes after the injection showed that the P and R waves were practically synchronous (figure 13). One and one half minutes later, however, the P waves again preceded the R waves, but by an interval of 0.03 to 0.08 of a second, and were partly fused with them (figure 14). The P and R waves were entirely separated three and one quarter minutes later, while the blood pressure had risen again to 40 millimeters of mercury (figure 15). Nineteen and one half minutes after the injection, the P waves had become negative (figure 16), and twenty-three and three quarters minutes after the injection, normal complexes were obtained, the P-R time being 0.10 of a second, the rate 167 per minute, and the blood pressure still 40 millimeters of mercury (figure 17). From this time on, the blood pressure rose gradually, the rate diminished somewhat, while the conduction (P-R) time showed no further change, and the electrocardiograms continued normal.

DISCUSSION.

The experiments which have been described demonstrate that during anaphylactic shock, caused by the intravenous injection of horse serum into sensitized dogs, disturbances of the heart-beat may occur which produce definite changes in electrocardiograms. These cardiac disturbances manifest themselves in several ways. The conduction of the stimulus of the heart-beat from auricles to ventricles may be definitely delayed or even occasionally prevented, causing in the electrocardiograms prolongation of the P-R time or the occurrence of P waves which are not followed by ventricular complexes. The ventricular contractions may become abnormal, yielding markedly altered ventricular complexes. These changes in the ventricular complexes are well defined in the electrocardiograms of three experiments and consist of a diminution of the R waves, a marked deepening of the S waves, and an exaggeration of the T waves, which become sometimes partly fused with the S waves. This change in the form of the ventricular portions of the curves was gradual and departed more and more from the normal after the anaphylactic shock until a maximum was reached, and then usually returned gradually to the form obtained before injection. These changes resemble closely those observed by Eppinger and Rothberger[10] in electrocardiograms of dogs, when a 20 per cent. silver nitrate solution was injected directly into the wall of the right ventricle, or when the limb of the atrioventricular bundle leading to the right ventricle was cut. When the silver nitrate solution was injected into the wall of the left ventricle, they obtained a distinctly different form of electrocardiograms. It seems evident, therefore, from their experiments that the side of the heart into which injections are made determines the character of the change in form of the electrocardiogram. As the changes in the curves of our experiments always resembled those of Eppinger and Rothberger in which the right side of the heart was injured, it seems possible that in our experiments the ventricular activity was disturbed by damage to the musculature, especially of the right ventricle, or to the path of con-

[10] Eppinger, H., and Rothberger, C. J., *Wien. klin. Wchnschr.*, 1909, xxii, 1910; *Ztschr. f. klin. Med.*, 1910, lxx, 1. Rothberger, C. J., and Winterberg, H., *Zentralbl. f. Herz- u. Gefässkrankh.*, 1913, v, 206.

duction in the right ventricle. The analogies which can be drawn between their curves and ours offer evidence for the possibility that the right side of the dog's heart is more affected in anaphylactic shock than the left, just as the right side of the rabbit's heart seems to be, as pointed out by one of us.[11] Still further evidence is given by our work on the rabbit during anaphylaxis.[12]

The development of a mechanism unusual in spontaneously beating hearts has already been mentioned. It occurred in experiment 11 (figures 13 to 17). The electrocardiograms indicate that the ventricles suddenly ceased to follow the auricles in the normal manner, but that the two parts of the heart contracted synchronously. This continued for several minutes, the auricles contracting either at the same time or very shortly before the ventricles, when the time which elapsed between the onset of auricular and ventricular systoles was not sufficient for the normal passage of the impulse. At first the P waves had a normal outline, but later the form of the P waves changed suddenly to a wave directed downward. A few minutes afterwards the electrocardiogram was again normal.

Such changes in electrocardiograms from the dog have been published by Rothberger and Winterberg.[13] After stimulation of the left accelerator nerve the P and R waves gradually approached each other and then became synchronous. Suspension curves of the auricles and ventricles, made simultaneously with the electrocardiograms, show that the auricles and ventricles contracted synchronously. Later on the normal relation of these waves gradually returned. They interpret this phenomenon, which was observed in a number of experiments, as the result of stimulation of the left accelerator nerve, and believe that the power of stimulus formation in the atrioventricular region is raised by the activity of the left accelerator, so that this region supersedes the normal region as the cardiac pace-maker. Thus atrioventricular autonomy is established, the point of origin of the heart-beat gradually shifting from the sino-auricular region to the atrioventricular region. These observations have been repeated and confirmed recently by Ken Kuré,[14]

[11] Auer, J., *loc. cit.*

[12] Auer, J., and Robinson, G. C., *loc. cit.*

[13] Rothberger, C. J., and Winterberg, H., *Arch. f. d. ges. Physiol.*, 1910, cxxxv, 559; see also figures 1 and 2.

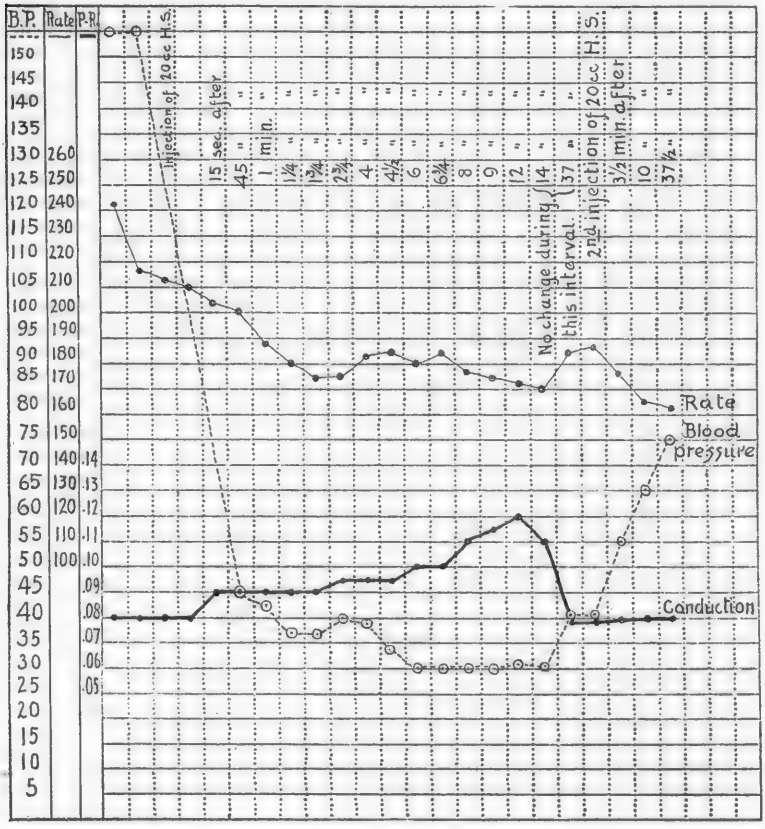
[14] Ken Kuré, *Ztschr. f. exper. Path. u. Therap.*, 1913, xii, 384.

who interprets his findings in a similar manner, although ordinary graphic and not electrocardiographic methods were used.

The phenomenon observed in our experiment is also to be interpreted as resulting from changes in the point of origin of the heart-beat. If the junctional tissue suddenly assumed the function of pace-maker, the auricles and ventricles would contract synchronously, and later, the point of origin having changed to the auricles, the auricular activity would precede the ventricular. The occurrence of the negative P waves is also accounted for by a junctional tissue as the origin of the heart-beat. This same type of change in the electrocardiogram has been met in our work on rabbits, as will be described elsewhere. It is not possible to say what relation these changes, which began eleven and one quarter minutes after the injection of the horse serum, had to the anaphylactic reaction. The fact that such changes occurred in the rabbit, in which no anesthetic was used, makes it unlikely that the occurrence of an atrioventricular rhythm was the result of etherization. Nor is it possible to see any definite relationship between its occurrence during anaphylaxis and after left accelerator stimulation.

The possibility that the cardiac disturbances are secondary to the marked fall in blood pressure must be considered. In order to determine the effect of a sudden lowering of blood pressure on the electrocardiogram of the dog, three experiments were performed in which the blood pressure was lowered by intratracheal injections of amyl nitrite, intravenous injections of sodium nitrite, and by cutting both the greater and lesser splanchnic nerves. The experiments were performed under the same conditions as those in which anaphylactic shock was produced, except that non-sensitized dogs were used. As the result of these procedures, rapid changes in blood pressure of 60 to 90 millimeters of mercury were obtained, without any definite changes in the electrocardiograms. The only change in conduction time, which was observed in these experiments, was a shortening which sometimes accompanied the fall in blood pressure, a change opposite to that seen in the positive anaphylactic experiments. The fact that a fall in blood pressure of 65 millimeters of mercury may occur without changing the cardiac mechanism, as indicated by the electrocardiogram, is illustrated by figures

18 and 19. Moreover, in several experiments in which anaphylaxis was produced, the drop in blood pressure was not followed by changes in the electrocardiograms. And when changes were observed, the relation between the fall in blood pressure and the cardiac



TEXT-FIG. 2. Curve constructed from the records of experiment 10. There were no changes in the shape of the complexes at any time.

disturbances was such as to render it unlikely that the former was the cause of the latter. Thus in experiment 8 the marked delay in conduction, which occurred one minute after the injection, returned

to normal, while the blood pressure remained at a level even slightly lower than that at which it was when the conduction time was most prolonged (text-figure 1). In this experiment and in several others, the heart was beating normally before the rise of blood pressure commenced. Another curve (text-figure 2), obtained from experiment 10, will make this lack of relationship between cardiac disturbances and low blood pressure still clearer. Here the blood pressure dropped from 155 to 45 millimeters of mercury within forty-five seconds after the injection of horse serum. Nevertheless, the conduction time rose only from 0.08 to 0.09 of a second and the electrocardiograms showed no alteration throughout the experiment. This answers an objection which could be made to our interpretation of text-figure 1: the abrupt drop of blood pressure and consequent anemia of the heart muscle cannot now be held responsible for the increased conduction time and for the change in the complexes such as text-figure 1 from experiment 8 shows, because an even greater drop of blood pressure (text-figure 2 from experiment 10) affected the conduction time but slightly and the shape of the electrocardiograms not at all. From the facts, therefore, that marked and sudden drops of blood pressure were not followed by any cardiac changes in some cases of anaphylaxis and in control experiments, and that the abnormalities in the electrocardiograms disappeared in several experiments before there was any rise in blood pressure, it seems legitimate to assume that the cardiac disturbances were not secondary to lowered blood pressure, but represent an independent phenomenon of the anaphylactic reaction.

In order to determine whether the central cardiac inhibitory mechanism played any part in the production of the cardiac disturbances of anaphylaxis, both vagi were cut in four experiments before the injection of horse serum. In addition, it seemed advisable to investigate this question, because changes in the heart-beat are produced by certain drugs which resemble those occurring in our experiments and which seem to be caused, at least in part, through the action of the drug on the vagus center. The partial dissociation so often seen following the administration of digitalis therapeutically, and the disturbances of the heart-beat, which have been ob-

served by Einthoven[15] and by Eyster and Meek[16] after the injection of morphin, disappear after atropin is administered or after the vagi are cut. In our experiments (Nos. 11, 12, 15, and 17) in which the vagi were cut, the effect of the injection of serum was apparently entirely similar to that observed in the other experiments, where the vagi were intact. In two of these experiments, Nos. 11 and 17, no definite alteration occurred in the heart-beat. In the third, partial heart-block developed (figures 10 to 12), while in the fourth, marked changes in the form of the ventricular complexes occurred. These were similar to those seen in other experiments in which the vagi were intact (figures 3 and 5). It can be stated, therefore, that the principal changes in the electrocardiograms during the anaphylactic reaction occurred both with the vagi intact and after the vagi had been cut. The central cardiac inhibitory mechanism thus does not play a part in the production of the cardiac disturbances which have been observed in the anaphylactic reaction of the dog.

The action of horse serum itself and ether, as causative factors of the cardiac changes described above, we excluded in control experiments on normal dogs where horse serum was injected or where the blood pressure was lowered by various means. No changes comparable to those observed during anaphylaxis were observed. We may also call attention to the experiments when horse serum was injected during the state of anti-anaphylaxis, which will be discussed below.

In eight anaphylaxis experiments the dogs were reinjected with twenty cubic centimeters of horse serum after they had partially recovered from the drop in blood pressure. None of them showed any effect on the blood pressure nor on the shape of the electrocardiogram, although the first dose had perhaps caused marked alterations in the form of the complexes, as in experiments 8, 9, 14, 15, and 16. In these experiments, therefore, each dog served as its own control as far as ether, horse serum, and anaphylactic phenomena are concerned. These experiments show that horse serum and ether *per se* do not cause cardiac disturbances of the kind we have described, and, secondly, they demonstrate that these changes

[15] Einthoven, W., and Wieringa, J. H., *Arch. f. d. ges. Physiol.*, 1913, cxlix, 48.
[16] Eyster, J. A. E., and Meek, W. J., *Heart*, 1912, iv, 59.

EXPLANATION OF PLATES.[17]

Plate 42.

Fig. 1. Experiment 8. Vagi intact. Electrocardiogram before injection. Conduction (P-R) time = 0.09 of a second. Blood pressure = 100 mm. of mercury. Rate = 212.

Fig. 2. Experiment 8. 1¼ minutes after injection. Conduction (P-R) time = 0.17 to 0.18 of a second. Blood pressure = 15 to 25 mm. of mercury. Rate = 161. Note delay in conduction.

Fig. 3. Experiment 8. 12 minutes after injection. Conduction (P-R) time = 0.09 of a second. Blood pressure = 10 to 15 mm. of mercury. Rate = 154. Note return to normal conduction without any increase in blood pressure.

Fig. 4. Experiment 15. Vagi cut. Electrocardiogram during injection. Conduction time = 0.09 of a second. Blood pressure = 115 mm. of mercury. Rate = 150.

Fig. 5. Experiment 15. 12¾ minutes after injection. Conduction time = 0.14 of a second. Blood pressure = 30 mm. of mercury. Rate = 167. There are marked changes in the form of the ventricular complexes and delay in conduction.

[17] Time is marked in 1/5 and 1/50 of a second.

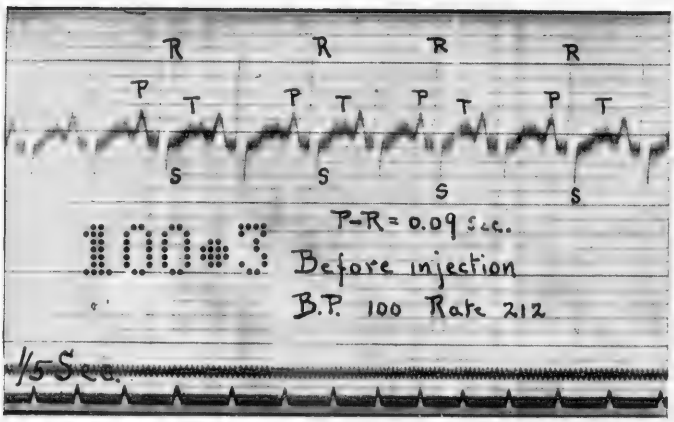

R
P
T
S
P-R = 0.09 Sec.
Before injection
B.P. 100 Rate 212
1/5 Sec.

FIG. 1.

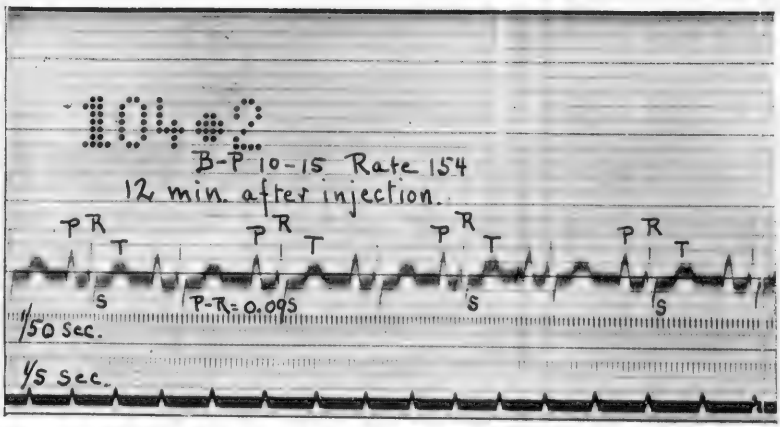

B-P 10-15 Rate 154
12 min. after injection.
P R T S
P-R = 0.095
1/50 Sec.
1/5 Sec.

FIG. 3.

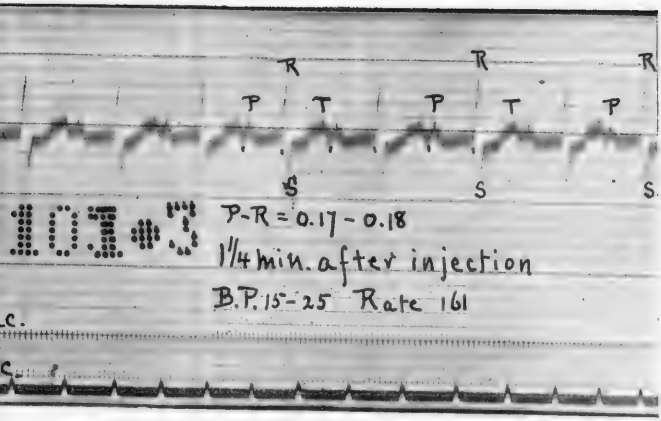
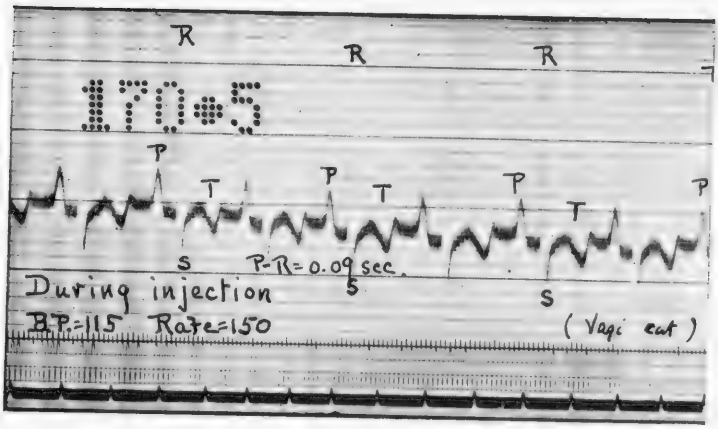

R
R
R
P
T
P
T
P
S
S
S
P-R = 0.17 - 0.18
1¼ min. after injection
B.P. 15-25 Rate 161

FIG. 2.


R
R
R
P
T
P
T
P
T
P
S
P-R = 0.09 sec.
S
S
During injection
B.P. 115 Rate 150
(Vagi cut)

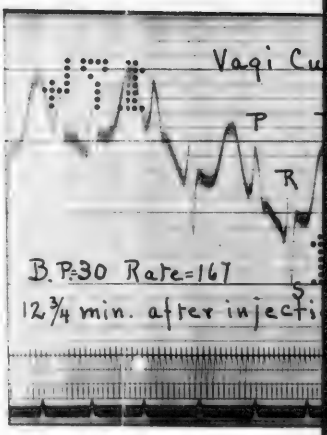
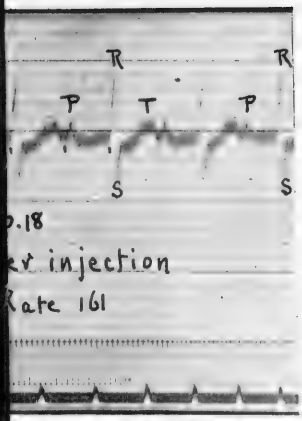
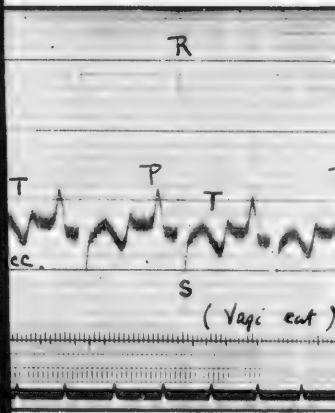

Vagi Cu
P
R
B.P. 30 Rate 167
12¾ min. after injecti

FIG. 4.


R
R
P
T
P
S
S
0.18
injection
Rate 161


R
T
P
T
S
(Vagi cut)

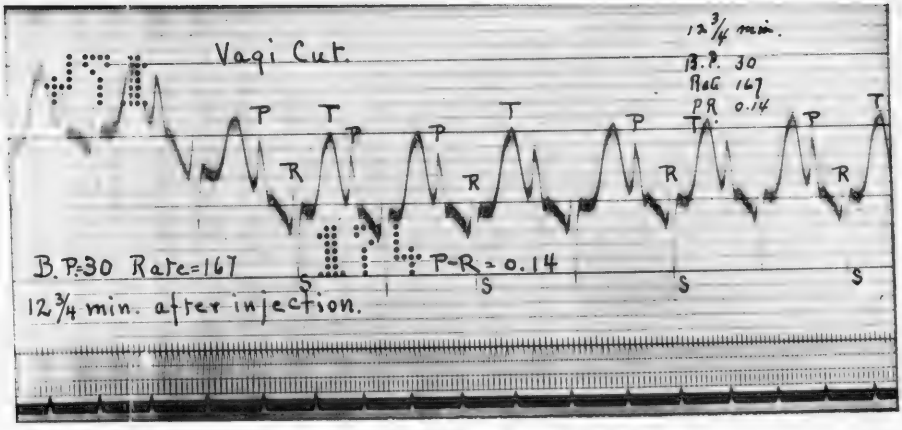


FIG. 5.

(Robinson and Auer: Disturbances of Heart-Beat in the Dog.)

PLATE 43.

FIG. 6. Experiment 1. Vagi intact. Electrocardiogram before injection. Conduction time = 0.08 of a second. Rate = 225.

FIG. 7. Experiment 1. 3 minutes after injection. Onset of partial auriculoventricular dissociation. Auricular rate = 267. One auricular beat in every eight is blocked. Conduction time varies from 0.12 to 0.28 of a second.

FIG. 8. Experiment 1. 9 minutes after injection. Partial auriculoventricular dissociation. Auricular rate = 233. One auricular beat in every four is blocked. Conduction time varies from 0.14 to 0.26 of a second. A change in form of the ventricular complexes similar to that in experiment 15 is seen.

FIG. 9. Experiment 1. 29 minutes after injection. Normal sequential beat. Conduction time = 0.12 of a second. Rate = 240. The form of the ventricular complex continues abnormal.

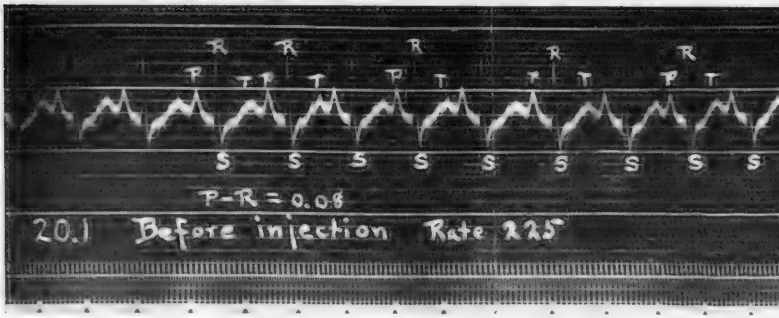


FIG. 6.

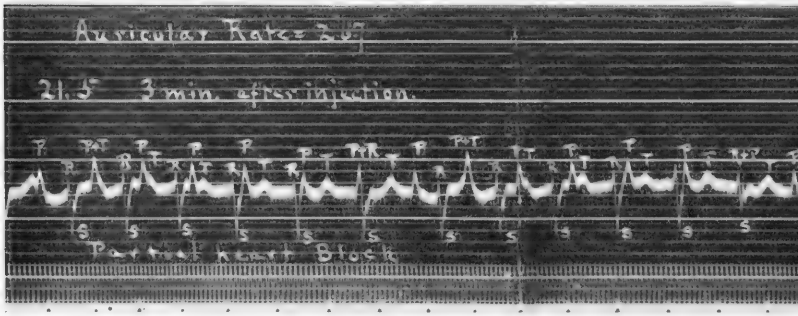


FIG. 7.

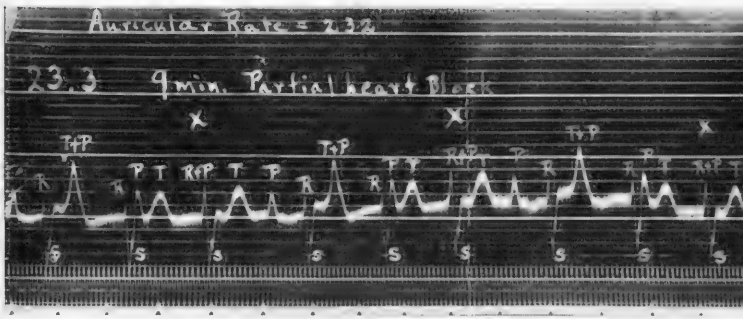


FIG. 8.

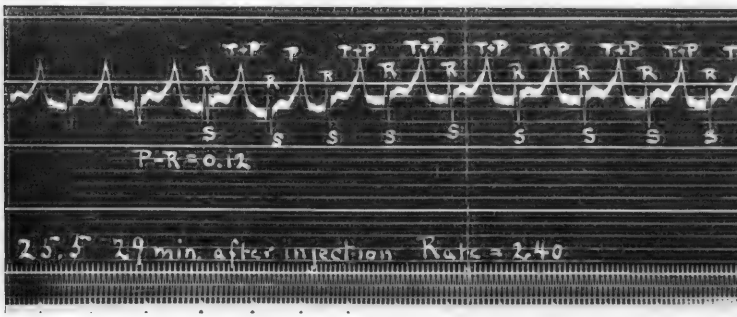


FIG. 9.

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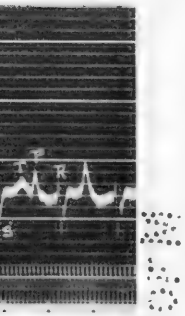
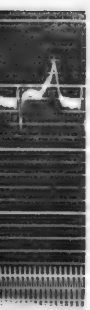
PLATE 43.





in the Dog.)

PLATE 44.

FIG. 10. Experiment 12. Vagi cut. Beginning of injection. Normal sequential beat. Conduction time = 0.08 of a second. Blood pressure = 145 mm. of mercury. Rate = 217.

FIG. 11. Experiment 12. 1¼ minutes after injection. Partial auriculo-ventricular dissociation. Blood pressure = 15 to 25 mm. of mercury. Auricular rate = 195. One auricular beat in every five is blocked. Conduction time varies from 0.14 to 0.28 of a second.

FIG. 12. Experiment 12. 2¼ minutes after injection. Sequential beat. Conduction time = 0.20 of a second. Blood pressure = 18 mm. of mercury. Rate = 187.

FIG. 13. Experiment 9. Vagi intact. 11½ minutes after injection. The auricles and ventricles are contracting synchronously. Blood pressure = 30 mm. of mercury. Rate = 148.

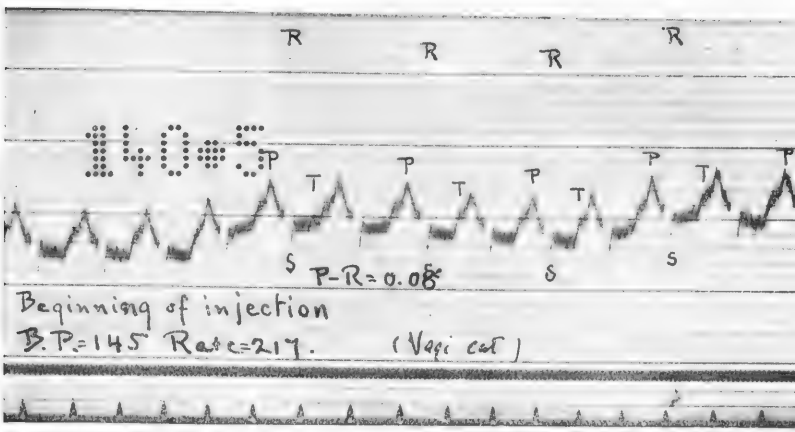

R
R
R
R
1405
P
T
P
T
P
T
P
T
S
P-R=0.08
S
S
Beginning of injection
B.P.=145 Rate=217. (Vagi cut)

FIG. 10.

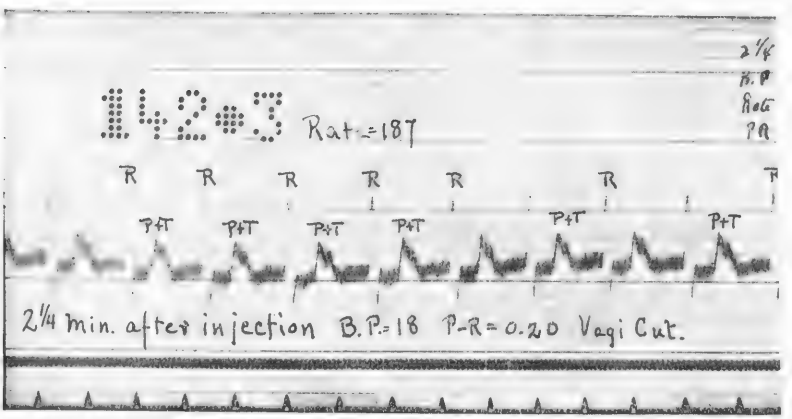

1420
Rate=187
R
R
R
R
R
R
P+T
P+T
P+T
P+T
P+T
P+T
2¼ min. after injection B.P.=18 P-R=0.20 Vagi Cut.

FIG. 12.

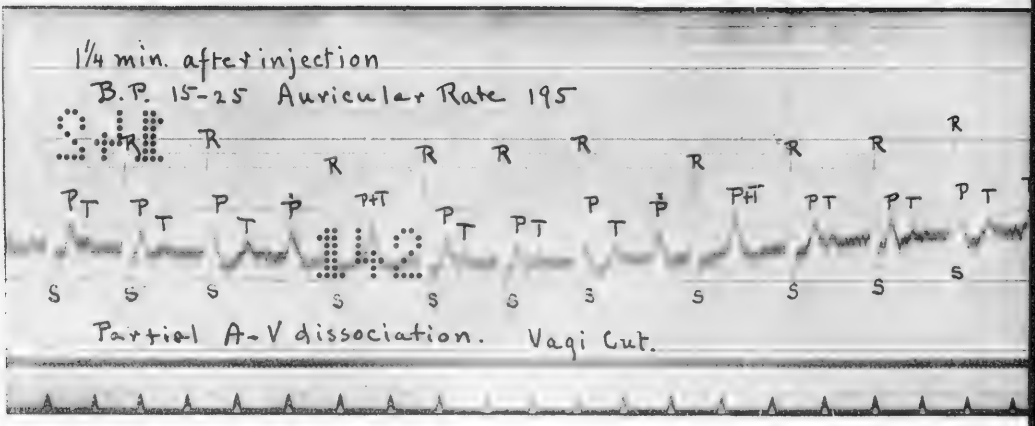


FIG. 11.

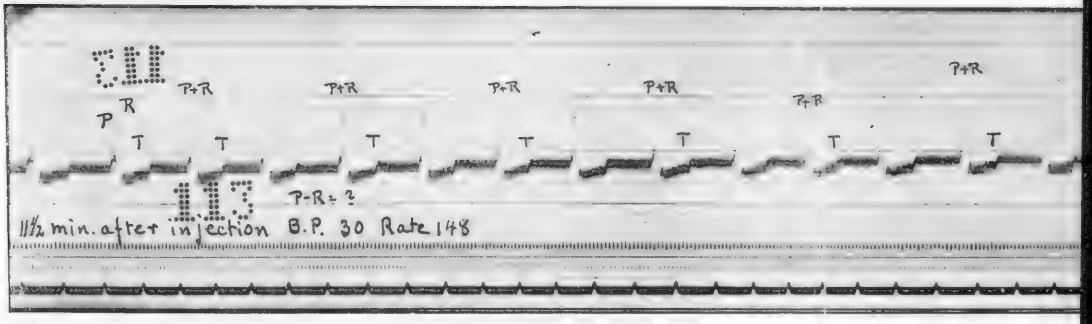


FIG. 13.

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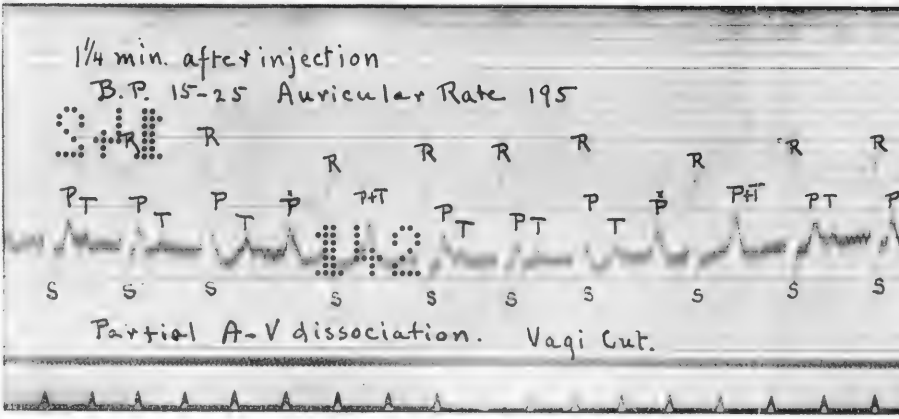


FIG. 11.

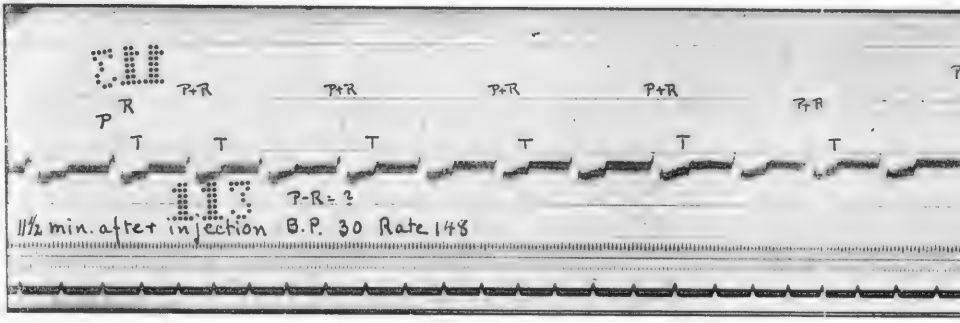
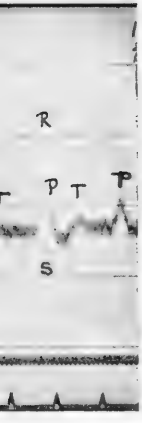
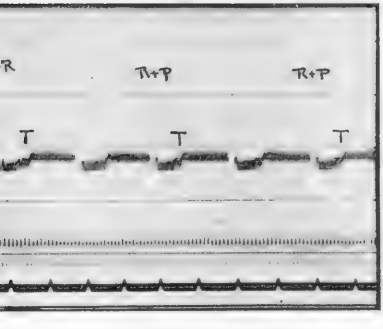


FIG. 13.

(Robinson





and Auer: Disturbances of Heart-Beat in the Dog.)

PLATE 45.

FIG. 14. Experiment 9. 12¾ minutes after injection. Ventricular contractions are seen following auricular contractions after an abnormally short interval. Blood pressure = 35 mm. of mercury. Rate = 152.

FIG. 15. Experiment 9. 16 minutes after injection. Conduction time = 0.07 to 0.08 of a second. Blood pressure = 40 mm. of mercury. Rate = 164. The electrocardiograms resemble closely the control curves.

FIG. 16. Experiment 9. 19½ minutes after injection. Conduction time = 0.06 of a second. Blood pressure = 40 mm. of mercury. Rate = 160. The P wave is inverted.

FIG. 17. Experiment 9. 23¾ minutes after injection. Conduction time = 0.08 of a second. Blood pressure = 40 mm. of mercury. Rate = 167. The electrocardiogram resembles closely that obtained before the injection, except that the R wave is broken and not so high. Before injection blood pressure = 135 mm. of mercury. Rate = 210. Conduction time = 0.09 of a second.

FIG. 18. Experiment 5. Vagi intact. Unsensitized dog. Electrocardiogram at onset of experiment. Conduction time = 0.11 of a second. Blood pressure = 120 to 125 mm. of mercury. Rate = 138.

FIG. 19. Experiment 5. After both splanchnic nerves had been cut and 2 c.c. of amyl nitrite had been injected into the trachea. Blood pressure = 55 to 60 mm. of mercury. Rate = 114. Conduction time = 0.10 of a second. No marked change is seen in the form of the electrocardiograms following the fall of 65 mm. of mercury in blood pressure.

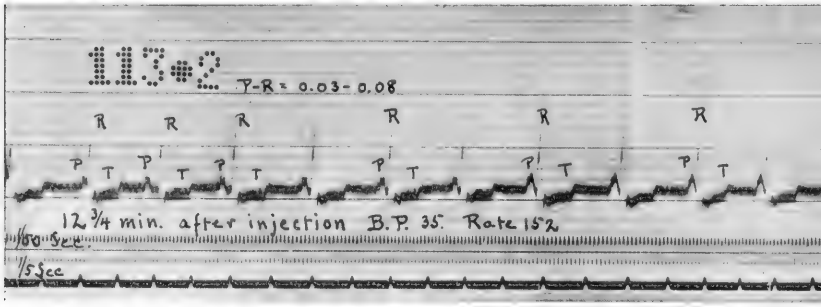


FIG. 14.

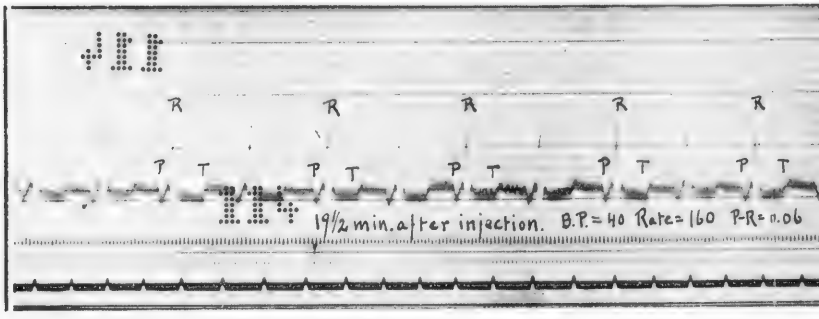


FIG. 16.

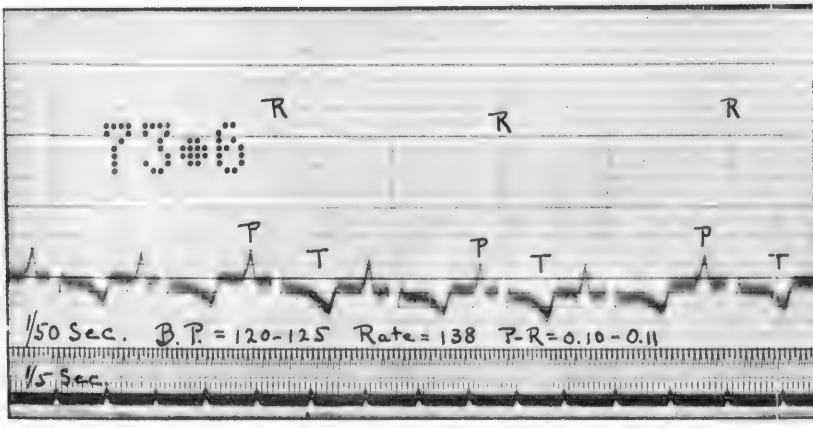
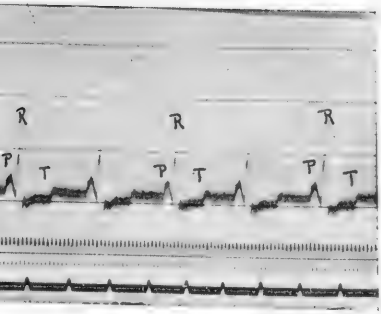


FIG. 18.



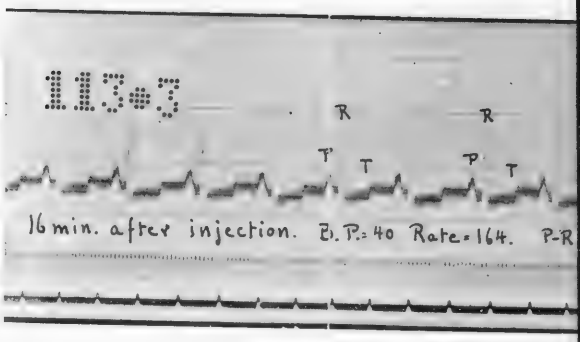


FIG. 15.

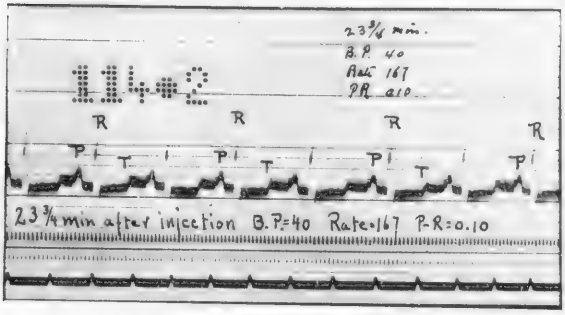


FIG. 17.

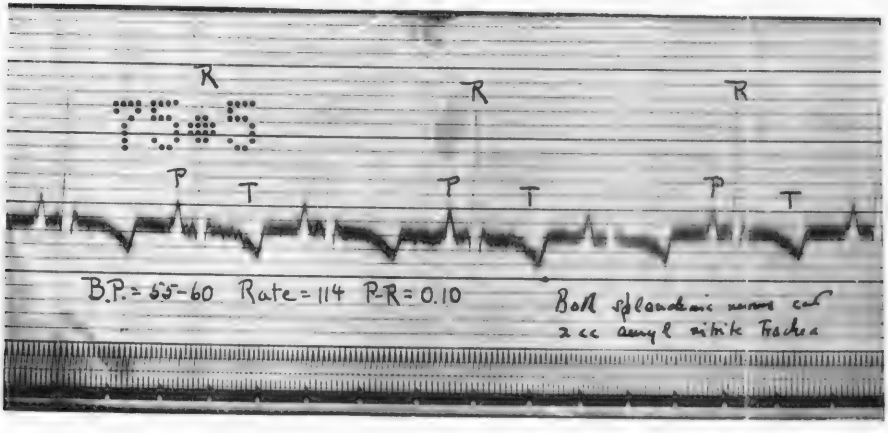


FIG. 19.

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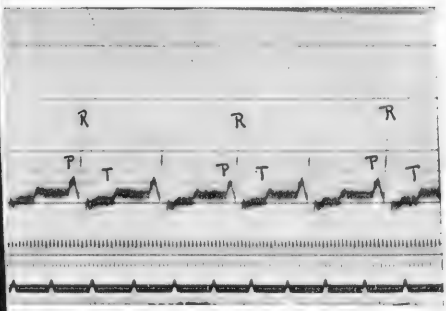


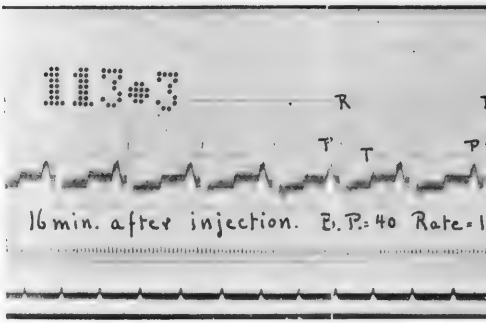


FIG.

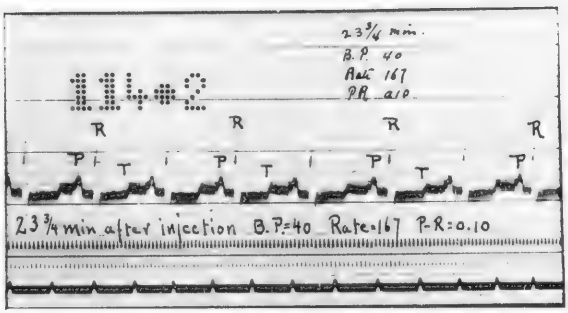
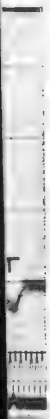


FIG. 17.



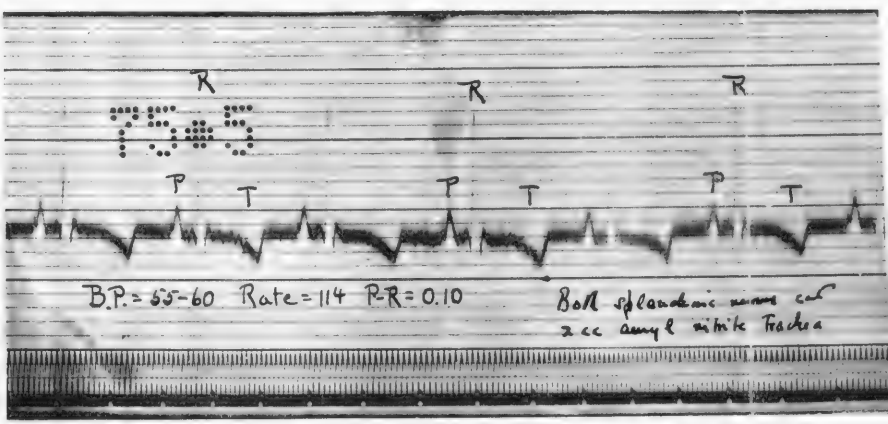
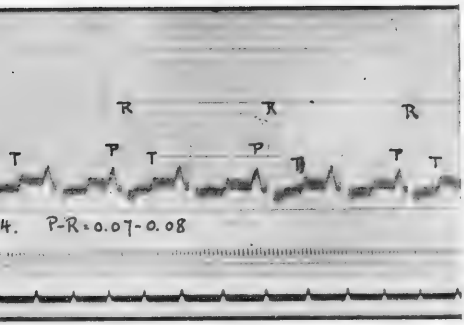


FIG. 19.



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(Robinson and Auer: Disturbances of Heart-Beat in the Dog.)

are of a true anaphylactic nature, since they are not obtainable during the anti-anaphylaxis induced by the first reinjection.

An interesting question, which these experiments bring up, is the possible part which the cardiac changes play in the production of the characteristic drop of blood pressure. It seems to us that the heart does not have a vital action in this respect, for a characteristic blood pressure effect may be obtained without any derangement of the cardiac beat. Thus Biedl and Kraus's main contention that the heart is negligible as a factor in the causation of the fall in blood pressure is probably correct.

The facts outlined above justify us, we think, in concluding that the cardiac changes noted during anaphylaxis in the dog are not of central origin, nor are they secondary to the drop in blood pressure or to the action of horse serum and ether *per se,* but that they are caused by an action on the peripheral cardiac tissue itself. What this action may be we shall not discuss at present.

CONCLUSIONS.

Anaphylactic shock in the dog, caused by the intravenous injection of horse serum into sensitized animals, may produce definite cardiac disturbances which are revealed by the electrocardiogram. These cardiac changes consist of disturbances in conduction of the heart impulses, abnormalities in the ventricular contractions, and other unusual disturbances of the mechanism of the heart-beat. They come on very quickly after the injection of serum and may be of short duration, and are not obtained during anti-anaphylaxis. They are not the result of the marked fall in blood pressure which occurs, nor does the central cardiac inhibitory mechanism play a part in their production. These cardiac disturbances apparently are a definite primary expression of anaphylactic shock in the dog. The right ventricle seems to be more affected than the left.

EXPERIMENTS ON THE CULTIVATION OF SO CALLED TRACHOMA BODIES.*

By HIDEYO NOGUCHI, M.D., and MARTIN COHEN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York.*)

Plate 46.

In 1907 von Prowazek and Halberstaedter[1] described certain cell inclusions in the conjunctival epithelia of persons suffering from trachoma. The inclusions were composed of coarse coccoid and more numerous minute granular bodies that were partially embedded in an irregular, indefinite, and homogeneous substance or matrix. The coarser bodies stained bluish and the minute ones reddish in Giemsa solution. When the scrapings from the conjunctiva showing the cell inclusions were introduced into the eye of orangoutangs there appeared after several days in the conjunctiva similar inclusions accompanied by a mild form of conjunctivitis. The two disappeared together. von Prowazek and Halberstaedter concluded that the inclusions are the etiological agents of trachoma; and von Prowazek has proposed for them the name of chlamydozoa.

This discovery was speedily confirmed, and the bodies were viewed as the etiological cause of trachoma when von Prowazek and Halberstaedter[2] detected similar inclusions in the conjunctiva in cases of uncomplicated blennorrhea neonatorum. Thus a discussion arose as to the specificity of the trachoma bodies, and Herzog advanced the theory[3] that under certain conditions the gonococcus undergoes transformation into small forms which, when accumulated within the protoplasm of epithelia, assume the appearance of so called trachoma bodies. Herzog believed that this transformation was accomplished by a rapid succession of transplantations of

* Received for publication, July 1, 1913.

[1] Halberstaedter, L., and von Prowazek, S., *Deutsch. med. Wchnschr.*, 1907, xxxiii, 1285; *Arb. a. d. k. Gsndhtsamte.*, 1907, xxvi, 44.

[2] Halberstaedter, L., and von Prowazek, S., *Berl. klin. Wchnschr.*, 1909, xlvi, 1110.

[3] Herzog, H., *Arch. f. Ophth.*, 1910, lxxiv, 520.

the gonococcus *in vitro,* and he stated that trachoma is caused by infection with the transformed or mutated gonococcus (his micro-gonococcus). The present authors[4] studied cell inclusions in various conjunctival affections, and came to view the trachoma bodies as a definite pathogenic organism which can bring about a fairly well defined clinical condition peculiar to itself. They regarded the occasional occurrence in gonorrheal conjunctivitis of the gonococcus and the trachoma inclusion bodies as accidental and coincidental; and they considered that this coincidence could not be explained by the transformation of the gonococcus into the trachoma bodies or that the two separate etiological factors operate to produce trachoma. Williams,[5] on the other hand, has come to regard the inclusion as degenerated forms of the Koch-Week's bacillus.

Hence it appears that the so called trachoma bodies have been treated as definitely living organisms by von Prowazek who regards them as a new protozoan; by Herzog who considers them a mutated gonococcus; and by Williams who regards them as degenerated Koch-Week's bacilli. Others consider them to be parasitic without definite orientation, and still others as products of degeneration, due to the invasion of cells by a still undetected etiological agent, analogous to those found in carcinoma, variola, rabies, and some other diseases. It has not been shown that the inclusions are the parasites themselves, and in order to settle this point proof must be brought that they are actually living, for which cultivation outside the body supplies the clearest evidence. What follows is in the nature of a preliminary report of our studies, in which the clinical part has been contributed by Cohen and the cultural by Noguchi.

Sources of Materials.—Three different groups of conjunctival affections were selected: (1) cases of conjunctivitis combined with so called trachoma body inclusions;[6] (2) cases of typical trachoma without the so called trachoma bodies; (3) cases of acute catarrheal conjunctivitis in which neither trachoma nor the cell inclusion affection occurred. To obtain the material the lid was inverted and carefully rinsed with sterile physiological salt solution, after which the

[4] Noguchi, H., and Cohen, M., *Arch. Ophth.*, 1911, xl, 1.
[5] Williams, A. W., *Arch. Ophth.*, 1913, xlii, 506.
[6] Cohen, M., *Arch. Ophth.*, 1913, xlii, 29.

surface of the conjunctiva was scraped with the edge of a sterile slide. The scraping was collected with a sterile platinum spatule and mixed in a tube with a small quantity of sterile ascitic fluid. In old trachoma cases where the conjunctivæ and tarsi were removed the tissues were immersed in a small amount of ascitic fluid to prevent desiccation. The materials were carried to the laboratory without delay.

Cultivation.—The usual culture media and methods were first employed without success. Then the procedure used for cultivating spirochætæ was adopted.[7] The scraping or piece of tissue was placed in a culture tube in which a piece of fresh sterile animal tissue (rabbit kidney) and a large quantity of sterile ascitic fluid were already present. The tube employed measures 1.2 by 20 centimeters, and the amount of ascitic fluid used is about ten cubic centimeters in each tube. After the inoculation a layer of sterile paraffin oil (two or three centimeters high) is poured upon the ascitic fluid and the tube is placed in an anaerobic jar. Usually two or three tubes are made with each specimen of scraping or tissue, and several specimens cultured on each occasion. Cultivation is conducted at 37° C.

The organism to be described was isolated in all cases of conjunctivitis with cell inclusions, and in one of three cases of trachoma without the inclusions. It was absent from all the control cases. The fact should be emphasized that the organism could not be cultivated at each trial, but at times repeated attempts were necessary for success. The failure with old cases of trachoma may be due to the removal of conjunctivæ and tarsi, so that further attempts at cultivation could not be made.

The tubes remain in the anaerobic apparatus about ten days, at which time films from each tube are made by removing the fluid with a sterile capillary Pasteur pipette. The air-dried films are fixed in methyl alcohol (Merck's reagent) for thirty minutes and stained with Giemsa solution for one hour. Films from cultures in which the inclusion bodies are growing show many extremely minute coccoid organisms, mostly in pairs, but some in heaps of varying numbers. The cultures contain, of course, ordinary bacteria, such

[7] Noguchi, H., *Jour. Exper. Med.*, 1912, xvi, 199, 211.

as staphylococci, streptococci, diplococci and certain bacilli; but the minute size of the inclusion bodies and their reddish stain enables one to recognize them without much difficulty.

The purification of the culture is the most tedious phase of the work, and much patience is required to accomplish it. The cultures usually contain many organisms growing profusely on the surface of the ascitic fluid agar, while the colonies of the inclusion organism are slow to develop and extremely minute. They do not attain a size easily visible to the naked eye, and as they are almost transparent are easily overlooked. They do not grow on the surface of the agar unless ascitic fluid or other suitable serum components are present. The medium found best suited thus far consists of two parts of slightly alkaline beef infusion agar and one part of ascitic fluid with 0.5 per cent. of glucose. To obtain colonies sufficiently isolated for fishing many dilutions of the original culture must be made; and inoculation may be made in the anaerobic apparatus or without it, but the former gives better results, since certain aerobic organisms are partially or completely prevented from developing. The minute organism is a facultative anaerobe which during the first two or three generations grows better in the absence of oxygen, while in later generations it is unaffected by the air.

In order to obtain pure growth a culture showing discrete, almost invisible colonies is selected, and by means of a fine platinum needle a single colony is touched and transferred to a new tube of medium containing a piece of sterile kidney and ascitic fluid. The sterile paraffin oil having been added, the tube is placed at 37° C. From now on the anaerobic apparatus is not employed. When transfers are made into various other media without the addition of fresh tissue the result is usually negative. For some generations the organism fails to grow on the surface of agar even when ascitic fluid and glucose are present.

After three or four days the fluid culture shows slight turbidity about the tissue, but no gross change takes place. Films made from the bottom of the tube and stained with Giemsa solution reveal the organism, usually in two main and many intermediate forms. The first form consists of rather large, lanceolate, or ovoid bodies, some occurring singly, others in pairs and groups or rosettes (figures 3,

4, and 5). The second variety are extremely minute, round or somewhat ovoid in shape, arranged usually in heaps or pairs (figures 17 and 18). The coarse forms take a blue, the finer a red stain. The two forms may be separate, but usually occur mingled in a single mass, the coarser occupying the outer and the minuter bodies the inner zone. A few coarse forms may lie among the minute ones, the two thus representing a typical trachoma body. Intermediate forms are always present, but they appear merely to constitute a stage during which the coarse forms are undergoing transformation into minute granules (figures 9, 10, 13, and 14). By starting with a single colony and following its development day by day it can easily be demonstrated that the different morphological elements are not due to contamination one with another. The coarse bodies predominate during the first two days, and are replaced by the minute ones. A comparison between the cultivated organism and the cell inclusions in corresponding developmental stages reveals a striking similarity (figures 1, 2, 6, 7, 8, 11, 12, 15, and 16).

Although when freshly isolated the organism grows best under anaerobic conditions, after several generations a good growth may be obtained in the presence of oxygen. During the anaerobic period the organism develops only in media containing animal protein, but after it has become aerobic it will grow without the addition of serum, although development is less free than when the latter is present. The colonies developed on the surface of the agar slant vary according to the degree of removal from the parent culture. Thus, during the anaerobic phase the colonies assume the appearance of minute, transparent, and almost invisible specks, whereas in the aerobic phase they are more distinct, though still extremely minute. In the depth of the agar they develop throughout the entire column within forty-eight hours as minute specks of greyish white color. No turbidity is produced in serum agar and no gas in glucose media. In neutral or slightly alkaline bouillon (beef infusion, peptone) the organism grows slowly in fine, powdery, slightly opalescent masses. The tissue at the bottom (if such has been added) becomes pale and grey. No odor is produced in any medium. The best medium is ascitic fluid tissue, covered with a layer of paraffin oil, in which the

organism grows well after forty-eight hours, the organisms remaining transplantable for a period of two months at 37° C. Blood is not hemolyzed.

Morphological Properties.—This organism is Gram amphiol and throws off the stain completely if decolorized long enough. It is distinctly stained by carbolized fuchsin or gentian violet, and only faintly by Loeffler's methylene-blue.

Various strains obtained from different cases present slight morphological deviations, and a strain obtained from one patient may appear somewhat coarser than that derived from another. The initial body forms may be arranged in rosettes or in irregular heaps, or even in short chains. The individuals in some strains are more round or oval in shape, and in others preponderatingly spindle-shaped, triangular, or crescentic. When the organism enters upon the elementary body stage some strains form much more minute granules than others. Similar variations occur among the cell inclusions which exist in cases of trachoma, blennorrhea neonatorum, and inclusion conjunctivitis.

Animal Inoculation.—Repeated attempts to produce the cell inclusions in various kinds of monkeys (Macacus and Papio) by means of pure cultures of this organism failed. In some instances a transient papillary conjunctivitis lasting about one week was produced, but the films showed only initial body forms, without cell inclusions.

SUMMARY.

1. An organism was isolated and studied in pure cultures from cases of conjunctivitis accompanied by the so called trachoma body inclusions, as well as from a case of old trachoma without inclusions.

2. This organism was not found in the cultures made from other forms of conjunctivitis in which the inclusions were absent.

3. The organism presents the morphological features characteristic of so called trachoma bodies. It undergoes an early transformation, during which the forms known as initial bodies appear, and a later change, during which forms resembling elementary granules arise, while certain intermediate forms between these occur simultaneously.

4. No definite cell inclusions could be produced in monkeys by inoculating pure cultures of the organism.

5. The cultivation of this organism from a case of trachoma without the cell inclusions, together with the previously ascertained fact that the inclusions can be produced in suitable animals by inoculating such material directly from a human case, suggests the possibility of the organism being still present in such cases of trachoma, although not in the form of cell inclusions.

6. The facts presented justify the statement that by a suitable method an organism resembling the various important stages of the trachoma bodies and totally distinct from the gonococcus has been obtained in a living condition capable of indefinite cultivation from cases of human trachoma and inclusion conjunctivitis. Whether the organism and trachoma bodies are identical cannot be positively stated at present. But the way is now opened to determine this point, as well as the specificity of the trachoma bodies.

EXPLANATION OF PLATE 46.

All the figures are from film preparations stained with Giemsa solution. Magnification × 1,000.

FIG. 1. Initial stage of the so called trachoma bodies in a conjunctival epithelium from a case of trachoma.

FIG. 2. Similar inclusion from a case of so called inclusion conjunctivitis.

FIGS. 3, 4, and 5. Initial stage of the cultivated organism, forty-eight hours old in ascitic fluid tissue media.

FIGS. 6 and 7. Intermediary stages of the trachoma bodies from a case of trachoma.

FIG. 8. Intermediary stage of similar inclusion from a case of so called inclusion conjunctivitis.

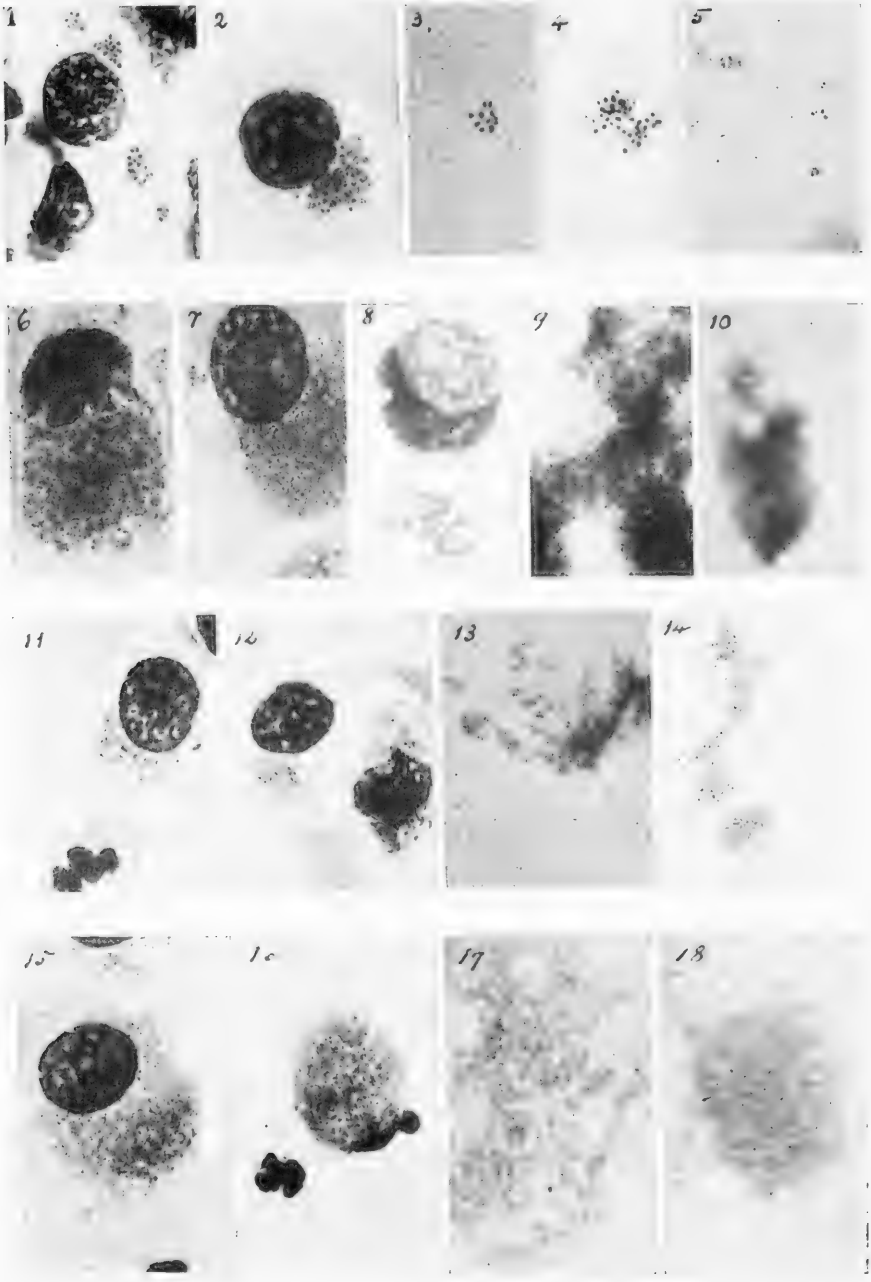
FIGS. 9 and 10. Intermediary stage of the cultivated organism, three to four days old in ascitic fluid tissue media.

FIGS. 11 and 12. Inclusions consisting of intermediary and elementary forms of the trachoma bodies, from a case of trachoma and one of inclusion conjunctivitis.

FIGS. 13 and 14. The cultivated organism showing stages where intermediary and elementary forms are mingled, four to five days in ascitic fluid tissue media.

FIGS. 15 and 16. Inclusions consisting exclusively of minute (elementary) granules from similar cases.

FIGS. 17 and 18. The cultivated organism which entered the stage corresponding to the elementary granules of the inclusions, seven days in ascitic fluid tissue media.



(Noguchi and Cohen: Cultivation of So Called Trachoma Bodies.)

THE TOXICITY OF GENTIAN VIOLET AND ITS FATE IN THE ANIMAL BODY.*

By JOHN W. CHURCHMAN, M.D., and LUCIUS FELIX HERZ, M.D.

(*From the Laboratory of Surgery of Yale University, New Haven.*)

Plates 47 and 48.

The observation of bactericidal properties possessed by gentian violet, and particularly of its affinity for pyogenic organisms, made it important to determine the toxicity of this substance and its fate in the animal body. A series of about seventy-five experiments has been done on dogs and rabbits with these points in view and the more important results will be reported in this communication.

INTRAVENOUS INJECTIONS.

For the study of intravenous injections of gentian violet rabbits were used. The dye in varying concentrations was injected into the ear vein. The cornea, conjunctiva, and mucous membranes of both mouth and lips immediately became a deep blue. The animals in some instances remained perfectly passive during the injection; but more often they became restive after a portion of the dye had entered the vein, their struggles suggesting that the material was irritating. This was, however, a transient manifestation, the animals becoming perfectly quiet and normal as soon as the needle was withdrawn. We were unable to discover why this symptom of irritation was present in some cases and absent in others; possibly the rate of injection was the determining factor.

The results of intravenous injections were of two kinds. In one group of animals the dye seemed to be absolutely without effect. The rabbits either remained quiet during the experiment or struggled somewhat towards its close. They usually sat about quite still for a short time after the experiment and then resumed their normal habits. Several of the animals in this group received enormous doses (ten cubic centimeters of a 1 to 200 solution), so that gentian

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violet in a dilution of about 1 to 2,500 was circulating in the vessels. Yet these animals remained alive and are still perfectly well a number of months after the experiment. The experiments proved conclusively that large amounts of gentian violet in the circulation may be borne by rabbits without any apparent harm whatever.

In another group of experiments, however, the animals died within a minute or so after the injection. In these cases the rabbits went into violent convulsions as soon as the injection was completed and died rapidly, often in opisthotonus. The autopsy in one or two cases showed acute edema of the lungs, and occasionally frothy sputum was discharged from the mouth before death. But these features were not constant; the autopsy was usually negative, aside from the staining of the tissues; and we are unable to explain death in these animals. That it was not due to the toxicity of the drug, in the ordinary sense of that word, seems likely from the rapidity with which death occurred and from the survival, without any symptoms, of a number of rabbits which received a dose as large or larger. That it was immediately dependent on the injection is equally evident. Possibly the strain on the circulation by the injection of such a large amount of fluid was one of the factors, though this would hardly account for the convulsions.

THE FATE OF THE DYE IN THE BLOOD.

The fate of the dye in the blood was studied by means of divided blood plates made from animals which had previously received intravenous injections of gentian violet. These experiments showed conclusively that blood withdrawn from animals so injected possesses the selective bactericidal power of the dye, but that this power disappears completely in about two hours. Figures 1 to 5 show the results of such an experiment. This animal received ten cubic centimeters of a 1 to 200 solution of gentian violet into the ear vein. Blood was then withdrawn from the heart immediately, and at the end of three quarters of an hour, one and three quarters hours, two and one quarter hours, and one week. With this blood, divided plates were so made that the upper half should have contained (provided the gentian violet remained unchanged in the blood) the dye in a dilution of 1 to 10,000,—a strength far in excess

of that needed for the selective bactericidal action. It will be seen that in the plate made immediately (figure 1) the usual selective action of the dye has manifested itself; and this is also true of the plate made after three quarters of an hour (figure 2), though here the reaction is not quite so sharp. In the later plates, however, the blood has lost entirely its selective bactericidal property (figures 3, 4, and 5).

It was thought that this disappearance of the dye might be due to a chemical reaction between the blood itself and the gentian violet. Two sets of experiments were done to test this point. In the first set, blood was withdrawn and gentian violet added to it in a test-tube. With this blood divided plates were made immediately, at the end of twenty-four hours, and at the end of one week. The selective power of the dye was uninfluenced by the presence of the blood, as can be seen in figures 6, 7, and 8. A similar set of experiments was also done with blood drawn into sodium oxalate, to prevent clotting, and then dialyzed against normal sodium chloride. The selective bactericidal power was still present at the end of a week (figures 9, 10, and 11). It is evident therefore that gentian violet injected intravenously into rabbits disappears from the blood in a short time, and that there is no similar loss of selective bactericidal power when the dye is simply allowed to remain in contact with blood *in vitro*. It is to be remembered that in speaking of the disappearance of the dye we refer to the disappearance of the selective bactericidal power, for it was by bacteriological and not by chemical tests that the presence or absence of the dye was determined.

STAINING OF THE TISSUES.

We have already referred to the fact that intravenous injection of gentian violet results in prompt and fairly deep staining of the visible mucous membranes. The dye also appears in the mucous membranes lining the gastro-intestinal tract. The visible membranes remain stained for about forty-eight hours, the stain disappearing slowly during this time. We have not followed the fate of the dye in the other organs. That the staining of the organs accounts in part for the disappearance of the dye from the circulation is obvious; the phenomenon is, however, chiefly due to rapid oxidation or reduction.

We have made a number of observations on the effect of the dye when applied directly to living tissues. The first studies were made by painting the tongue of dogs and rabbits with strong solutions of the dye. Frozen sections showed that penetration had occurred through the thickness of the mucosa down to the muscularis. Similar observations were made on the bladder, the mucosa of which is readily stained by injecting the dye into that viscus through the urethra. In like manner, the synovial membranes may be deeply stained by intra-articular injections. Just how irritant the dye is cannot at present be stated. On the tongue it seems to be without irritant effect; in the bladder, in the very strong solutions used, there was a good deal of inflammatory reaction.

An attempt was made to treat a diphtheria carrier by painting the pharynx and tonsils with gentian violet. In view of the fact that the diphtheria bacillus is constantly gentian-positive[1] and that the mucous membranes may be stained to their depths by painting the dye on the surface, this seemed to be a rational procedure. The results were somewhat suggestive, but an unexpected obstacle was met in the secretion of the mucous glands of the pharynx. This material lay over the surface of the pharynx in a thin slimy layer, which was renewed by fresh secretion as rapidly as it was removed, and which prevented intimate contact between dye and mucous membrane as effectually as a layer of grease would have done. The observation was instructive as indicating some of the difficulties which will have to be met in attempting to treat infections of mucous membranes by local applications. This obstacle is being borne in mind in attempts which are now being made in the New Haven Hospital to apply some of the findings as to the bactericidal properties of gentian violet to the treatment of septic arthritis.

EXPLANATION OF PLATES.

Plate 47.

Fig. 1. Divided plate made with blood withdrawn from the heart of an animal which had received 10 c.c. of a 1 to 200 solution of gentian violet intrave-

[1] In the first communication on the selective bactericidal action of gentian violet the diphtheria bacillus was reported as gentian-positive (Churchman, J. W., *Jour. Exper. Med.*, 1912, xvi, 221). This statement was based on the study of only a few strains. It has recently been found in a study of a large number of strains that the organism is constantly gentian-positive (Guthrie, C. C., personal communication).

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

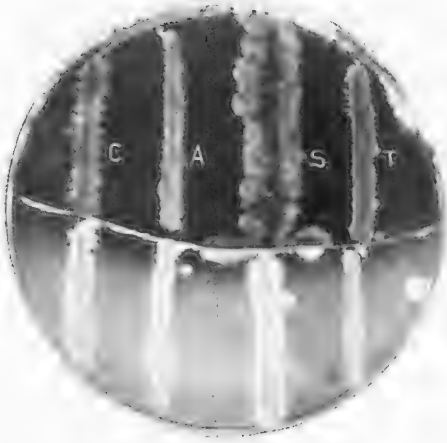

FIG. 5.

FIG. 6.

(Churchman and Herz: Toxicity of Gentian Violet.)

FIG. 7.

FIG. 8.

FIG. 9.

FIG. 10.

FIG. 11.

(Churchman and Herz: Toxicity of Gentian Violet.)

nously. Blood withdrawn and plate made immediately after injection. Organisms used were *B. coli, B. subtilis, M. aureus,* and *B. typhosus.*

Fig. 2. The same as figure 1, except that blood was withdrawn and the plate made three quarters of an hour after injection.

Fig. 3. The same as figure 1, except that blood was withdrawn and the plate made one and three quarters hours after injection.

Fig. 4. The same as figure 1, except that blood was withdrawn and the plate made two and a quarter hours after injection.

Fig. 5. The same as figure 1, except that blood was withdrawn and the plate made one week after injection.

Fig. 6. Divided plate made with gentian violet and blood mixed in a test-tube. Plate stroked immediately with *B. coli, M. aureus, B. subtilis,* and *B. typhosus.*

Plate 48.

Fig. 7. Divided plate made with blood and gentian violet which had been allowed to stand in contact in a test-tube for twenty-four hours. Note that the gentian violet is present in strong enough dilution to inhibit *B. typhosus,* though still without effect on *B. coli,* illustrating the greater sensitiveness of the former organism to the dye.

Fig. 8. Divided plate made with blood and gentian violet which had been allowed to stand in contact in a test-tube for one week.

Fig. 9. Divided plate made with blood passed into sodium oxalate and then mixed with gentian violet. Plate made and stroked immediately.

Fig. 10. Divided plate with blood passed into sodium oxalate and then allowed to stand in contact with gentian violet in a test-tube for twenty-four hours.

Fig. 11. Divided plate made with blood drawn into sodium oxalate and then allowed to stand in contact with gentian violet in a test-tube for one week.

THE COMPARATIVE VIABILITY OF PNEUMOCOCCI ON SOLID AND ON FLUID CULTURE MEDIA.*

By L. J. GILLESPIE, Ph.D.

(From the Hospital of The Rockefeller Institute for Medical Research, New York.)

It has long been recognized among bacteriologists that the viability of the pneumococcus on artificial culture media varies. Kruse[1] asks why it is that the pneumococcus will sometimes refuse to grow upon a medium prepared in the standard manner with especial regard to the proper reaction. Experience in this laboratory has shown that no difficulty is found in obtaining abundant growths of pneumococci in broth prepared as will be described later, provided that enough infected material is introduced, but that sometimes the necessary quantity is very large, especially if large volumes of broth are inoculated. It has also been observed that the amount necessary to produce a growth in broth may be larger than that necessary to produce a growth on agar. We have found that a loop of infected blood is generally sufficient to set up a growth in five cubic centimeters of broth, but if this be inoculated into half a liter of broth, no growth occurs. In order to obtain a growth in a half liter or liter flask, it is usually necessary to inoculate with five cubic centimeters of young cultures. A series of studies was undertaken to obtain more accurate data in regard to the observed facts and, if possible, to shed some light on the essential nature of these phenomena. The following paper forms a brief report of these studies.

METHODS.

The broth used for all experiments was a beef infusion containing 0.5 per cent. of sodium chloride and 1 per cent. of Witte's peptone (Kahlbaum), sterilized intermittently in streaming steam for short periods. The reaction was 0.6 per cent. acid to phenolphthalein. The agar used in all experiments contained 0.5 per cent.

* Received for publication, July 12, 1913.

[1] Kruse, W., Allgemeine Mikrobiologie, Leipzig, 1910, 158.

glucose. Sheep serum glucose agar contained 20 per cent. of sheep serum sterilized separately at a low temperature. All the broth employed in each experiment, including that used for dilutions, was of a single lot. In making inoculations into broth, the material was introduced carefully in the middle of the surface, care being taken not to infect the sides of the tube.

DIFFERENCE OF VIABILITY OF THE PNEUMOCOCCUS WHEN INOCULATED INTO BROTH AND ON AGAR.

The method employed was to inoculate varying amounts of pneumococcus culture into broth and on sheep serum glucose agar, to determine the amount necessary in each case for growth to occur. Thirty-three cultures were so tested. The strains tested included representatives of groups I and II, as described by Dochez,[2] as well as *Streptococcus* or *Pneumococcus mucosus*. The cultures were obtained directly from the heart's blood of inoculated animals. All the strains tested were of high virulence, not more than 0.000,001 of a cubic centimeter of the broth culture being required to kill a mouse. In each case one loop of heart's blood from the mouse was inoculated into five cubic centimeters of bouillon. After twenty-four hours' growth, progressive dilutions were made from this culture as follows: A series of tubes was taken, each tube containing five cubic centimeters of bouillon. To the first was added 0.5 of a cubic centimeter of the culture and a mixture made by rotating the tube, care being taken to produce as little foam as possible. This is called dilution tube 1. Of this mixture, 0.5 of a cubic centimeter was taken and added to the second tube containing five cubic centimeters of bouillon and mixed as before. This was called dilution tube 2. In a similar manner successive dilutions were made, in each case 0.5 of cubic centimeter of the mixture being added to the succeeding tube to form the next lower dilution. Now from each one of these dilution tubes, one drop was added to a tube containing five cubic centimeters of bouillon, and a mixture made as before, and one drop was added to a tube of slanted agar and distributed over the surface. A similar inoculation of one drop from the undiluted culture was also made in broth and on agar. These latter

[2] Dochez, A. R., and Gillespie, L. J., *Jour. Am. Med. Assn.*, 1913, lxi, 727.

inoculations are numbered 0 in the protocols. The tubes were then observed daily for at least five days to determine whether or not growth had occurred. If growth occurred on the agar, it was evident in twenty-four hours. In the broth, if growth occurred at all, it was usually heavy in twenty-four to forty-eight hours, but in a few instances the growth was apparent only after seventy-two hours. Whenever it occurred, it was abundant. In no case was there observed only a slight initial growth without further development. The following table shows a typical result obtained in one of these experiments.

TABLE I.

Pneumococcus I.

	0	1	2	3	4	5	6	7	8
Dilutions	−	+	+	−	−	−	−	−	−
5 c.c. broth	+	−	−	−	−	−	−	−	−
Slant agar	+	+	+	+	+	+	+	−	−

From this experiment it is apparent that many more organisms were required to start the broth cultures than were necessary to produce a growth on agar. By calculation it was determined that about 1,000,000 organisms viable on agar were required to produce a growth in five cubic centimeters of bouillon. Similar results were obtained with all the strains tested, though the results were not quite so striking in every instance. In four of the experiments the results were somewhat discordant, growth being obtained in certain of the higher dilutions when not occurring in the lower.

It has been found convenient, in estimating the comparative viability of pneumococci in broth and on agar, to speak of a broth agar difference, meaning by this the difference in the numbers of tubes in which growth occurs, when the technique as described above is employed. For instance, when growth occurs in broth only in tube 1, while on agar growth occurs in all the tubes up to and including No. 8, the broth agar difference is spoken of as seven tubes. From such differences have been calculated the approximate numbers of cocci viable on agar necessary to start a growth in broth. Table II shows the results obtained in the thirty-three experiments so expressed.

TABLE II.

Determinations by Means of the Broth Agar Difference of the Numbers of Pneumococci Viable on Agar Which Are Necessary to Start a Growth in Broth.

Broth agar difference, in tubes.	Number of cocci viable on agar required to start a growth in broth.	Number of strains tested.
7	10,000,000	1
6	1,000,000	7
5	100,000	6
4	10,000	3
3	1,000	6
2	100	3
1	10	2
0	1	4
−1	0.1	1

EFFECT OF REPEATED PASSAGE ON ARTIFICIAL MEDIUM.

It was soon found that the broth agar difference became less and even finally could be made to disappear, if, instead of employing cultures directly from the animal body, growths from broth after repeated and continuous passage through this medium were used. In one experiment, for instance, the broth agar difference of a culture obtained directly from the animal body was five tubes. After two transfers through broth the broth agar difference was only four tubes; after five transfers, the difference was only three tubes; after fifteen transfers only two tubes; and after seventeen transfers the broth agar difference had entirely disappeared. In other cases so large a number of transfers was not required to bring about this change. Repeated reinoculation on agar had essentially the same effect.

EXPERIMENTS UNDERTAKEN TO EXPLAIN THE BROTH AGAR DIFFERENCE.

The possibility at once suggested itself that the reason why growth from a small number of organisms is more likely to occur on agar than in broth is that the agar medium is better suited to growth of pneumococci and contains substances which are favorable to its growth. Comparative tests showed no differences between glucose agar and sheep serum glucose agar. It seemed possible, however, to test this hypothesis by retaining only the broth as a culture medium and still obtaining an arrangement that would

reproduce to a large extent the solid surface of the agar. This was done as follows: Narrow strips of Schleicher and Schuell's No. 597 filter paper were taken and folded twice, so that when placed in test-tubes a flat shelf would be formed, supported by two legs resting on the bottom of the test-tube. A number of such strips were prepared and placed in test-tubes and sterilized. Then into the tubes broth was carefully poured, so as to wet all parts of the paper and to fill the tubes to within about two centimeters of the transverse shelf. Inoculations were then made into these tubes by placing a drop of the diluted culture on the filter paper shelf, instead of mixing it with the bouillon, as was done in the tubes containing no filter paper. A large number of experiments were performed to compare the broth agar difference as determined in the usual way with the broth filter paper difference. Table III gives the results obtained in this series of experiments. All the cultures employed were obtained directly from the animal body, as in the first experiments described.

TABLE III.

No. of experiment	1	2	3	4	5	6	7	8	9	10	11	12
Broth agar difference, in tubes.	5	6	2	6	2	2	2	1	0	—1	—1	—1
Broth filter paper difference..	5	6	2	6	2	2	2	1	0	—1	—1	—1

It is evident that when the growth on filter paper in broth is compared with the growth in broth alone, the same differences, though to slightly less extent, are seen as are found when growths on agar and in bouillon are compared.

In such experiments the imitation of a solid medium by the filter paper expedient is not perfect. Not all the inoculated material remains on the paper and, on the other hand, in some instances growth may not extend to the broth below and so the occurrence of growth may be overlooked. These experiments indicate clearly that chemical differences alone between the two sorts of medium cannot be responsible for the phenomenon under consideration.

It was next suggested that the difference might be due to difference in available oxygen, the supply of oxygen being much better at the surface of a solid medium than in the depths of the fluid. To test this hypothesis inoculations were made into tubes of melted

glucose agar, which were then allowed to harden, as well as on the surface of agar tubes. The results obtained were compared with the results obtained by making corresponding inoculations into bouillon. In this case the organisms in the depths of the solid medium were under at least as unfavorable conditions as regards oxygen supply as were the organisms in the fluid medium. No essential differences were found between the growths when the inoculations were made into the depths of solid medium from those when they were made on the surface; in both cases growth occurred from inoculation with equally small numbers of organisms and from inoculation with much smaller numbers than were necessary to produce growth in broth. Comparative tests were also made between growths in broth in the ordinary way and broth incubated in an atmosphere of oxygen. The oxygen had no apparent influence in inducing growth to occur from higher dilutions. It therefore seems that the slight difference in oxygen supply on the surface of solid medium and in the depths of liquid medium is not sufficient to explain the broth agar difference.

DISCUSSION.

From the experiments described it would seem that neither chemical differences in composition of culture medium nor differences in available oxygen are sufficient to explain why in one case on solid medium a few bacteria are able to live and multiply and, in the other case, in liquid medium they fail to multiply and die.

A possible explanation is that the organisms in some way assist each other in their growth when they are closely approximated, and when they become scattered in fluid medium this favoring action is absent. It is possible that in their metabolic processes something is produced which is favorable to further multiplication, and in fluid medium this is diffused and diluted. Some years ago certain writers concluded from experiments on viability and on rate of reproduction, that microörganisms sometimes produce substances which favor their multiplication. Wildiers[3] apparently was the first to make this inference. His studies were made on yeasts. Among others who made similar inferences should be mentioned

[3] Wildiers, E., *La cellule,* 1901, xviii, 313.

Rahn,[4] who found that the time required for cell division, as determined for *Bacillus fluorescens liquefaciens* by plate counts, decreased for a while after growth started, to rise later as the culture grew old. The criticism generally made, however, against such conclusions[5] is that traces of poisons (copper and the like) may be present in ordinary culture media, and that these may exert a sufficient effect on very small numbers of bacteria to produce the phenomenon noted.

A second possibility is that such phenomena occur only during adjustment to a new environment, and that local irregularities of diffusion play a part. It may be conceived that when large numbers of organisms are introduced into fluid medium only a few happen to receive (through diffusion) a sufficient supply of nutrient material without experiencing too abrupt a change of environment.

CONCLUSIONS.

1. Pneumococci, when freshly isolated from the body, are able to live and multiply when a small number of them are inoculated into a small amount of broth. If, however, the inoculations are made in large amounts of broth, many more bacteria must be inoculated in order that they may grow.

2. It requires much smaller numbers of pneumococci to start a growth on agar than are required to start a growth in broth.

3. This predilection for solid medium disappears when the bacteria are grown for some time outside the body.

4. This phenomenon is not dependent on differences in chemical composition between the two media employed or on the presence of more available oxygen in one case than in the other.

5. It is probably dependent entirely on physical differences in the two kinds of media, and bears some relation to the differences in possibilities for diffusion in the two media.

[4] Rahn, O., *Centralbl. f. Bakteriol., 2te Abt.*, 1906, xvi, 417, 609.
[5] Kruse, W., *loc. cit.*, p. 167.

STUDIES ON FERMENT ACTION.

VIII. The Toxicity of Some Proteoses.*

By JAMES W. JOBLING, M.D., and SOLOMON STROUSE, M.D.
(*From the Nelson Morris Memorial Institute for Medical Research, Chicago.*)

In a previous article (1) we showed that the products of digestion of pneumococci by the ferment of leucocytes were capable of producing immune bodies when injected into the rabbit. By analogy with the digestion of other proteins these products should have consisted mainly of proteoses; for we have shown by our studies on the proteolytic action of leucoprotease (2) that leucocytic ferments as used in our experiments split mainly to the proteose stage. We then attempted to carry the experiments further in order to separate, if possible, the individual cleavage products and to test their biological action in the same way as was done by Jobling and Bull (3) with *Bacillus typhosus*. Jobling and Bull showed that the toxic element of *Bacillus typhosus* resided in the primary proteose fraction resulting from digestion with leucoprotease. It was important to attempt to fractionate the products of digestion of pneumococci by leucoprotease in order to determine whether the same fraction is toxic as with the typhoid bacillus. Perhaps the variations in the clinical course of certain diseases can be brought into relation with variations in the response of the bacteria to body ferments; or, in other words, the primary proteose derived from *Bacillus typhosus* may be toxic, and the same chemical compound from pneumococci may be non-toxic.

Attempts to pursue this line of investigation were begun, but the technical difficulties of securing pneumococcus protein free from mixtures of culture medium in sufficient quantity to carry on exhaustive experiments made it advisable to use other protein for preliminary experiments.

Witte's peptone, as is well known, contains a variable proportion of proteoses as well as of peptone and amino acids, and it was

* Received for publication, July 17, 1913.

decided to separate the various components and to make biological tests of the toxicity of the proteose fractions. It has been shown that the injection of Witte's peptone produces symptoms similar to those of anaphylactic shock, and much work has been done to elucidate the problems of anaphylaxis by means of the study of the chemistry of the reaction with Witte's peptone. As anaphylaxis will be discussed in this paper only as the phenomenon occurs in the course of our experiments, the literature on the subject will not be reviewed. The exhaustive study of Zunz (4) contains a complete bibliography. However, the conception that anaphylaxis and immunity are merely different stages in the same process makes it necessary to attach importance to studies attempting to define in terms of chemical formulæ the nature of any biological phenomenon. Most studies on the relation of protein digestion to immunological problems will be neglected, but we shall briefly review some of the more important contributions bearing directly on the question of the toxicity of proteoses.

De Waele (5) gave peritoneal injections of serum into sensitized guinea pigs, and at the height of anaphylactic symptoms, he withdrew the peritoneal exudate. This exudate on injection into normal guinea pigs produced acute symptoms resembling those of anaphylaxis. The exudate after removal of protein gave Millon's and the biuret reaction which were not present in the serum injected. Consequently, he argued, proteolysis had occurred in the sensitized guinea pig and the symptoms of anaphylaxis were due to protein digestion products.

Biedl and Kraus (6) and others have shown that analogies exist between the condition of anaphylaxis and intoxication by Witte's peptone. Vaughan and Wheeler's (7) method of treating protein resulted in hydrolysis. The separation into a non-toxic portion insoluble in alcohol and a toxic alcohol-soluble portion perhaps may be compared to the separation of proto- and heteroalbumose by the method of Pick (8). The work of Wells (9), of Pick and his co-workers (8) shows that the effect of digestion of albumen on its sensitizing and toxic properties varies with the nature of the ferment and the time of digestion; in other words, it depends on what substances result from digestion. Hartoch and Ssirenskij (10) treated serum protein with trypsin and produced substances toxic for rabbits at the first injection. The symptoms produced were similar to those of anaphylactic shock. Abderhalden and Kämpf (11) found that the portion of peptone precipitated by ammonium sulphate was toxic for guinea pigs. Friedberger (12) claimed that the toxicity of Witte's peptone was due to "primary proteose." During the course of our work Zunz (4) published his comprehensive report on the relation of anaphylaxis to proteoses, but in his studies no especial importance was attached to primary intravenous injection of the various proteoses. His protocols, however, show that control injections of all the substances were made, and that toxic symptoms arose only from the thioalbumose fraction.

Probably the most successful method of separating the various proteoses from a digestion mixture is that of Pick, but even with this procedure, according to Zunz (13), it is not a question of "chemically defined substances," but only of "groups, complexes, or combinations of proteoses." In the first experiments a 10 per cent. solution of Witte's peptone was used, its toxicity tested by injection into the jugular vein of guinea pigs, and then it was submitted to the following chemical procedures. Any free protein was removed by acidifying with acetic acid and boiling. The filtrate was neutralized, filtered after standing, and then the filtrate was treated with an equal amount of saturated ammonium sulphate. The precipitate (primary proteoses) was used in the experiments or further divided as follows: It was dissolved in a small amount of water, to which two parts of 95 per cent. alcohol were added. The heteroalbumoses are precipitated by this method, and the protoalbumoses remain in the alcohol. The protoalbumoses were removed from the alcohol by distillation at low temperature under diminished pressure. The filtrate remaining after half saturation of the original peptone solution was saturated with ammonium sulphate crystals and the precipitate (secondary proteoses) obtained. In some instances the thioalbumoses were first precipitated by two thirds saturation with ammonium sulphate. Throughout the work each step was repeated in order to purify the products. For most of the experiments the material was not thoroughly dried, nor was it free from traces of ammonium sulphate. The exact dosage cannot be given with precision, especially since a certain amount of material is always lost by filtering. In sufficiently large doses ammonium sulphate is not without toxic effects on guinea pigs, but this was controlled by a series of experiments to be described subsequently.

Experiment I.—1 c.c. of 10 per cent. Witte peptone solution was injected into the jugular vein of a small guinea-pig. The animal developed symptoms of anaphylactic shock—dyspnea, convulsions, etc.—and died within a few minutes. Autopsy showed dilated lungs.

0.1 c.c. produced symptoms (dyspnea, restlessness).

The primary proteoses from this solution were dissolved in water so that 1 c.c. was equivalent to 2 c.c. of the original peptone solution. Of this 1 c.c. injected into the jugular vein of a guinea pig caused immediate death. Autopsy showed lungs and stomach distended.

0.4 c.c. produced symptoms, but the animal survived.

Of the secondary proteoses an equivalent of 4 c.c. of the original peptone solution produced immediate toxic symptoms, with recovery. The symptoms with this injection were more convulsive than those obtained with the primary proteoses. A larger dose caused immediate death, but the autopsy showed no dilated viscera. The lungs were the seat of many small hemorrhages.

Heteroalbumoses.—Doses equivalent to 8 c.c. and to 12 c.c. of original peptone solution were without effect on intravenous injection into guinea pigs.

Protoalbumoses.—The equivalent of 3 c.c. of original peptone solution produced immediate anaphylactic symptoms with death in five minutes. Autopsy showed typical distention of lungs.

From this experiment the suggestion is derived that both proteose fractions of Witte's peptone are inherently toxic. Of the two components of the primary proteoses, that precipitable by alcohol does not seem toxic, while that soluble in alcohol is decidedly toxic. The symptoms and autopsy findings resulting from the injection of secondary were not exactly identical with those produced by the primary proteoses.

Experiment 2.—This differed from the preceding experiment only in that each of the various fractions was prepared and laid aside until all were ready, and the injection of the peptone solution and of the proteose fractions took place at the same time. The same results were obtained. The protoalbumose produced immediate marked symptoms, the heteroalbumose no symptoms, and the secondary albumose convulsions, prostration, death. At autopsy the lungs were retracted, the stomach and colon distended, and the peritoneal surface of the stomach and intestines injected.

These experiments were repeated with constant results so that they can be accepted. In order to rule out the possible harmful effect of the ammonium sulphate, an experiment was performed with fractions, all of which had been dialyzed for forty-eight hours. The toxic effects of the primary proteose fraction and of the protoalbumose fraction were verified. The secondary proteose fraction produced immediate severe symptoms, with recovery. In the undialyzed material the method of procedure would result in this fraction containing more sulphate than the other fractions, and it is important to find that the purified substance still causes toxic symptoms. In this experiment the heteroalbumose fraction produced death, this being the only time in our series that symptoms were produced by this portion. Perhaps, however, the intensity of the toxic symptoms of secondary proteose were increased by the ammonium sulphate present in the undialyzed material, and the small

pulmonary hemorrhages may have been due to the salt. The injection of the dialyzed material resulted in typical anaphylactic death, with typical autopsy, whereas an injection of a small amount of ammonium sulphate likewise produced convulsions and death. The autopsy on the last guinea pig showed numerous small hemorrhages in the lung, so that undoubtedly some of the symptoms following injection of the undialyzed secondary proteoses were due to ammonium sulphate.

In order to see whether proteoses from other sources than Witte's peptone had the same effects an experiment was performed with casein. A 1 per cent. solution of casein (technical, Merck) in 0.1 per cent. sodium carbonate was digested with leucoprotease for eight days at 37° C. The digestion mixture was then treated exactly as above outlined to separate the primary and secondary proteoses. The amount of primary proteose was comparatively large, while the amount of secondary proteose was small. Intravenous injections of these substances purified, in both cases produced marked symptoms and death. In the case of the primary proteoses the symptoms and autopsy were typically anaphylactic. With the pig dying from the injection of secondary proteoses there were marked convulsions and death in four minutes. The lungs were distended and somewhat hemorrhagic.

Experiments were also performed with the digestion products from egg albumen treated for one week with leucoprotease, and the toxicity of primary and secondary proteoses was studied. With the egg albumen definite toxic symptoms were produced by the primary proteose, but no marked symptoms with the secondary proteose, although the latter animal died in eighteen hours. In another experiment, however, definite symptoms and death were produced by the secondary proteoses. Further splitting of the proteoses derived from casein and from egg albumen was not attempted because of the small amounts of material.

TABLE I.

Injections.	Death.	Symptoms. No symptoms.	Symptoms. Recovery.
Witte's peptone	4	0	3
Primary proteose	4	1	2
Secondary proteose	6	1	2
Protoalbumose	3	0	1
Heteroalbumose	1?	3	1

Table I summarizes the results of injection of the various proteoses from all sources.

DISCUSSION.

In any discussion of the toxicity of proteoses it must be emphasized, as was stated in the text, that we are not dealing with chemical entities. In all probability the "groups" which are precipitated by various strengths of ammonium sulphate are not constant, but contain chemical substances grading one into the other. In order to fix biological properties on any one of these fractions the experiments would have to be absolutely conclusive and constant. In our work we obtained results pointing to toxic properties in both primary and secondary proteoses. Of the primary proteoses that group precipitated by alcohol seems to possess the least toxic effect.

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STUDIES ON FERMENT ACTION.

IX. A Note on the Relation between Lysis and Proteolysis of Pneumococci.*

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In our work on the immunity problems of pneumococcus infections in relation to proteolysis of the pneumococcus we had been accustomed to consider that in a test-tube containing organisms undergoing digestion we could estimate the amount of proteolysis by the degree of clearing up of the bacterial suspension. Chemical examinations proved that this is generally true; but in one experiment in which we used various preservatives, we found marked discrepancies between the appearance of the tubes and the amount of digestion as measured by formol titration. Since the technique involved in the study of bacterial ferments and of the action of other ferments on bacterial bodies is by no means simple it seemed worth while reporting the series of experiments undertaken to throw some light on this point of the problem.

EXPERIMENT I.

The Effects of Preservatives on Autolysis of Pneumococci. Twenty-Four Hours at 37° C. All Tubes Contain Pneumococci in Salt Solution.

Tube.	Preservative.	Gross appearance.	Formol titration in c.c. N/10 sodium hydrate.
1	Toluol	Very cloudy	0.48
2	Chloroform	Slightly cloudy	0.33
3	Ether	Slightly clear	0.40
4	None (pneumococci heated at 100° C.)	Very cloudy	0.50

In this experiment the tube containing ether as a preservative was clearer than any other but showed less digestion as measured

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by formol titration than the tube containing toluol, or the one with heat-killed pneumococci.

The amount of digestion with autolyzing pneumococci is very small, and in order to get more comparable results leucoprotease was used as a ferment.

EXPERIMENT II.

All Tubes Contain the Same Amounts of Pneumococci and Leucoprotease. Digestion for Forty-Eight Hours at 37° C.

Tube.	Preservative.	Gross appearance.	Formol titration.
1	Toluol	Cloudy	1.70
2	Chloroform	Cloudy	1.45
3	Ether	Clear	1.00

This shows more intensely than the first experiment the marked disagreement between the clearing and proteolysis, at least as measured by formol titration. The tubes in which ether was used show the greatest amount of solution of bacteria without corresponding comparisons of ferment action. This suggests at once that ether acts as a solvent for the lipoids of the bacterial body, permitting solution of the protein, and at the same time possibly inhibits ferment action. This suggestion is strengthened by the fact that the tube containing chloroform (also a fat solvent) shows the next degree of clearing.

EXPERIMENT III.

Digestion for Forty-Eight Hours at 37° C.

Tube.	Pneumococci.	Preservative.	Gross appearance.	Formol titration.
1	Dried with alcohol	Toluol	Cloudy	1.9
2	Dried with alcohol	Chloroform	Cloudy	1.8
3	Dried with alcohol	Ether	Clearest	1.8
4	Dried with alcohol and ether extracted	Toluol	Clear	2.17
5	Dried with alcohol and ether extracted	Chloroform	Clear	1.98
6	Dried with alcohol and ether extracted	Ether	Clear	1.90

In order to test out this idea a relatively large amount of pneumococci were dried by alcohol dehydration and then halved. One half was extracted with ether in the Sohxlet apparatus for five days. Then each half was suspended in water, leucoprotease was added, and each was divided into three equal parts, according to the above protocol.

This experiment shows that the clearing up of bacteria in a digestion mixture depends on other elements than the ferment. The pneumococci which had not been extracted with ether, when treated with leucoprotease and different preservatives cleared up in relation to the preservative, (1) ether, (2) chloroform, (3) toluol, while the amount of digestion was practically the same. With the same organisms extracted with ether, no differences in the gross appearance of the tubes could be made out after digestion with leucoprotease, although in this experiment there were rather distinct differences in the amount of digestion. The interpretation of this experiment confirms the suggestion above mentioned that the preservative acts in dissolving the bacteria by taking up the lipoids, while it may at the same time directly inhibit the ferment action on the bacteria.

This is the simplest explanation of the phenomenon and does not consider at all the question of the relation of the preservative to antiferment. If it is true it should be possible to substitute some similar protein for the bacteria, and repeat our results. Casein (technical, Merck) is a protein which contains a certain amount of lipoid substance and is in a measure analogous to bacterial protein. The effects of ether, chloroform, and toluol on the digestion of casein by leucoprotease should be similar to what has been outlined for pneumococci. Many such digestion experiments were performed, and in all there was marked clearing of the flask containing ether, less clearing of that containing chloroform, and practically none in that containing toluol. But the amount of digestion measured in various ways bore no relation to the physical appearance of the flask. With the casein experiments digestion was measured at times by total incoagulable nitrogen, at times by formol titration; in no instance did digestion under ether exceed that under toluol. Further proof that clearing is due to solution of

fatty substances rather than to proteolysis is seen in the large amount of these substances seen in the ether layer. Also casein, like pneumococci, when previously extracted with ether and then submitted to proteolysis no longer shows the marked changes in physical appearance.

SUMMARY.

Bürgers, Schermann, and Schreiber (1) studied "Auflösungserscheinungen von Bakterien," and concluded that ferments were probably not the main factor in such lysis. Kantorowicz (2) in studying antiferment and bacteriolysis came to the opinion that there was an antiferment in the bodies of bacteria which prevented digestion. This antiferment was likewise an antibody to serum bacteriolysins. But Kantorowicz used as a standard of ferment action merely the clearing up of the bacterial suspension.

Our report shows that lysis of pneumococci may be independent of ferment action, and that it is not correct to assume that clearing up of a bacterial suspension even in the presence of a proteolytic ferment indicates proteolysis.

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